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and
SECOND CONFERENCE ON MUSCULAR CONTRACTION

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Consulting Editor
DAVID A. NACHMANSOHN

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* This series of papers is the result of the *Second Conference on the Physicochemical Mechanism of Nerve Activity* held by The New York Academy of Sciences, Section of Biology, October 2 and 3, 1958. See *The Physico-Chemical Mechanism of Nerve Activity*, Vol. XLVII, Art. 4, based on the conference held by the Academy February 8 and 9, 1946.

† This series of papers is the result of the *Second Conference on Muscular Contraction* held by The New York Academy of Sciences, Section of Biology, October 3 and 4, 1958. See *Muscular Contraction*, Vol. XLVII, Art. 6, based on the conference held by the Academy February 15 and 16, 1946.

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PHYSICOCHEMICAL MECHANISM OF NERVE ACTIVITY

INTRODUCTORY REMARKS

David A. Nachmansohn
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Twelve years ago The New York Academy of Sciences published its first monograph on the physicochemical mechanism of nerve activity.[1] Several contributors to that publication have contributed to this one.

Many lively differences of opinion characterized the earlier monograph. This is as it should be. As Leonor Michaelis once said to me, "Science would be dull if all scientists would agree." Scientists are passionate people with strong convictions, and it seems only natural that their emotions affect their scientific judgment sometimes more than they realize. It is interesting to note that only a few years after publication of that monograph many problems looked quite different, and that views then proposed with great emphasis became rapidly obsolete. Today many people would find it difficult to understand the passion that some of the questions discussed then aroused. An important factor in the change has been, of course, the rapid progress of science in many directions.

In the twelve years that have passed, momentous developments have taken place, events that are rapidly changing the world situation. The power of science and knowledge has become obvious, not only to the leaders of our Western civilization, but to the majority of men all over the world. For a long time only a limited number of intellectuals were aware of the revolution that has taken place in physics since the turn of the century, but now the atom and hydrogen bombs, the sputnik, and similar by-products of this revolution have shaken the foundations not only of our society but of humanity as a whole. The impact of these developments on the various civilizations of the world will and must be profound, indeed, much greater than we are able to realize or evaluate at present.

However, for us scientists the technical, social, cultural, and political implications of the progress that has been made are not the only exciting and stirring aspects. For us the paramount importance of the advance of physics and chemistry has been the ever-increasing possibility of a better understanding of nature and of the world we live in that has resulted from this progress. For the biologist in particular, completely new horizons have been opened that justify the hope of achieving one of his ultimate aims, namely, to understand the living cell on a molecular level in terms of physics and chemistry. The two principal topics of this monograph, namely, the mechanism of nerve and muscle activity, are fields that have long attracted the passionate interest of many scientists, because investigation in them seems to offer a reasonable hope of achieving this aim. The past decade has seen impressive progress in both fields. The use of intracellular micro-electrodes for the study of electric manifestations, the availability of radioactive material for measuring ion movements, improved methods for very rapid recording of heat production, to cite but a few examples, have yielded much new information about the physical events. Electron microscopy and X-ray diffraction studies have provided new pictures of ultrastructure and, particularly in the case of muscle, have revolutionized our concept of the elementary process. Developments of biochemical aspects were just as striking and spectacular. The elucidation of metabolic pathways, the rapid progress of protein and enzyme chemistry, and the analysis of molecular forces acting in enzymes and their relation to function have advanced our knowledge of the chemical and molecular basis of cellular function at a very rapid rate.

Several months ago I participated at a conference on ion transport in Saclay, the great French center for nuclear studies. At the closing session Francis Perrin, High Commissioner of France for Atomic Energy, made an interesting comment in the course of a remarkable address. The first half of our century, he said, has seen a miraculous rise of physics and chemistry. He expressed his firm belief that during the second part of the century the really exciting developments would take place in biology, in our understanding of life processes. I hope that this monograph will contribute to the field, not only by providing new and valuable information, but by stimulating new and fruitful developments.

Reference

1. THE PHYSICO-CHEMICAL MECHANISM OF NERVE ACTIVITY. 1946. Ann. N. Y. Acad. Sci. **XLVII(4)**: 375–602.

Part I. Physical and Chemical Aspects of Nerve Impulse Conduction

ION MOVEMENTS DURING NERVE ACTIVITY

Andrew F. Huxley
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There are several different ways in which the electric activity of a nerve fiber depends on movements of inorganic ions. In the first place, activity at one point on a fiber stimulates the regions ahead by means of local-circuit currents flowing forward in the axis cylinder, out through the membrane, and back in the external fluid. The current in the external fluid is clearly carried by a straightforward process of conduction in an electrolyte, and the same thing is almost certainly true of the current in the axis cylinder. Evidence for this is given by the three following facts: (1) that the longitudinal current obeys Ohm's law fairly closely (Hodgkin and Rushton, 1946; Rashbass and Rushton, 1949); (2) that the specific resistance of the axoplasm is about what would be expected for an aqueous solution of the ions that are known by chemical analysis to be present; and (3) that the mobility and diffusion coefficient of the internal K^+ are close to those in aqueous solution (Hodgkin and Keynes, 1953). The ions in question are partly inorganic—notably potassium—but organic ions are also concerned in some cases at least. Thus, there are considerable quantities of dicarboxylic amino acids in crustacean nerve (Silber, 1941; Lewis, 1952); these are also present in squid nerve, but here the predominant anion appears to be isethionate (Koechlin, 1955). Phosphate esters are also likely to carry an appreciable fraction of the current in all kinds of nerve.

A second way in which ion movements are important is in the maintenance of the resting potential. As is well known, Bernstein (1902) suggested, on the basis of the dependence of the demarcation potential on temperature, that the resting potential is a concentration potential, due probably to potassium ions. The ability of K ions to cross the membrane that is required on this theory has been amply demonstrated in recent years both by observing net movements (Boyle and Conway, 1941) and by tracer measurement of K exchange in the resting state (Krogh, 1946, and Keynes and Lewis, 1951*a*, among others). Although these downhill movements of K are clearly an important factor, they are not the only means by which positive charge is carried outward to maintain the resting potential. On the one hand, Cl ions probably act in the same way (Boyle and Conway, 1941; Hodgkin and Katz, 1949*a*); Bernstein himself was aware that the Cl^- concentration ratio was in the right direction, but he appears to have thought (1912, page 99) that an emf due to chloride ions should add to that due to K^+, while in fact it is the currents carried by the various ionic species that are additive, the p.d. across the membrane being the same for all. On the other hand, there are also "active" movements of ions (Na and K), which we may regard as the third kind of ion movement of importance in nerve activity. These active movements, in which the net flux of the ion is in the direction that requires work to be done against its electrochemical gradient, have been much studied by tracer methods, and they are clearly essential for the long-term maintenance of the resting potential and therefore of electrical excitability. This, however, appears to be the only respect in which they (or any other process depending directly on metabolic energy sources) are necessary for excitation since, in many cases, abolition of active transport by anoxia or metabolic inhibitors does not prevent a nerve from propagating impulses (for example, Hodgkin and Keynes, 1955) while, in cases where excitability is lost, it may be restored by raising the membrane potential with applied current (Lorente de Nó, 1947).

The fourth type of ion movement is the one most directly linked with the characteristic activity of excitable tissues: the generation of an action potential. This again consists in downhill movements, but is characterized by rapid changes in permeability in response to change of membrane potential. The general idea is again originally due to Bernstein (1902), who postulated that the action potential consisted in a disappearance of the resting potential due to a general increase in permeability, allowing all kinds of ions to enter or leave the axoplasm freely. This form of the hypothesis became untenable when it was found that the interior of a nerve fiber becomes electrically positive with respect to the external fluid at the peak of a spike (Hodgkin and Huxley, 1939, 1945; Curtis and Cole, 1940, 1942), an observation that led to the demonstration (Hodgkin and Katz, 1949*a*; Hodgkin and Huxley, 1952*a*, *c*) that the spike in a squid axon was indeed due to a rise in membrane permeability, but one that was highly specific for Na ions during the rising phase. The inward movement of Na ions down their concentration gradient is thus enabled to make the interior of the fiber positive, the potential nearly reaching the value for a sodium concentration potential. The falling phase was shown to be brought about by a subsequent drop in the permeability to Na^+ and a rise in that to K^+. A similar role for Na has also been demonstrated in frog muscle (Nastuk and Hodgkin, 1950), frog myelinated nerve (Huxley and Stämpfli, 1951), and cardiac muscle (Draper and Weidmann, 1951), but in crustacean muscle it seems more probable that the charge is carried inward by Ca^{2+} (Fatt and Katz, 1953; Fatt and Ginsborg, 1958).

The time course of the permeability changes was investigated (Hodgkin *et al.*, 1952; Hodgkin and Huxley, 1952*a*, *b*, *c*, *d*) by the "voltage clamp" method, originally devised by Cole (1949) and Marmont (1949). A set of equations was fitted to the data (Hodgkin and Huxley, 1952*d*) describing the permeability changes and the resulting currents as functions of membrane potential and time. Theoretical action potentials were then computed by numerical methods from these equations, and good agreement with experimentally recorded action potentials was obtained as regards shape, velocity, membrane conductance change (Cole and Curtis, 1939), Na entry and K loss (Keynes, 1951; Keynes and Lewis, 1951*b*), and some subthreshold phenomena. This agreement encouraged us to think that the formulation we had used was substantially correct, but the scope of the computations was limited by the fact that an automatic computer was not then available, and the manual computation was very laborious. Since then, further cases have been solved on automatic digital computers, and the remainder of this article will be devoted chiefly to a discussion of them. As a preliminary, however, a summary of Hodgkin and Huxley's (1952*d*) formulation of the permeability changes will be given.

Summary of Equations

In the first place, it was found that, for a squid axon in sea water, the permeabilities to K and Na ions could be measured by the contributions to membrane conductance made by the movement of these ions. The justification for this was that each of these conductances, defined as the current carried by the ion, divided by the difference of membrane potential from the value where this current was zero, for example, $g_{\mathrm{Na}} = I_{\mathrm{Na}}/(V - V_{\mathrm{Na}})$, was found to have the same value immediately after a sudden shift of membrane potential as before it, that is, the conductance had a definite value at every instant. This is a purely empirical justification for the use of conductance as the relevant variable; it probably has no fundamental significance, since it was not found to hold good when the axon was in a low-sodium solution.

Second, the permeability changes appeared to be governed by membrane potential and not, for instance, by membrane current, since the time course of the conductance changes was the same for a given sequence of membrane potential changes even if the current was drastically altered by changing the external sodium concentration.

The time courses of the conductances when the membrane potential was made to change stepwise are illustrated in FIGURE 1, which also shows the manner in which mathematical expressions were fitted to the time courses. For example, the potassium conductance was found to increase in an S-shaped curve when the membrane potential was suddenly reduced, and to return along an exponential decay when membrane potential was restored. This behavior was fitted by defining a variable n, which obeys first-order kinetics, with rate constants which depend only on membrane potential. The quantity n^4 consequently behaves in a similar way to g_{K}, as shown in FIGURE 1, *C*. Sodium conductance was found to behave in the same way, except that its rise was quicker and that, if the depolarization was maintained, the conductance fell slowly toward a low level. This behavior was fitted satisfactorily by the quantity m^3h, where m and h each obey first-order kinetics; the process is illustrated in FIGURE 1, *D*, *E*, *F*, and *G*.

The equations are summarized below. The numerical values chosen by Hodgkin and Huxley (1952*d*) and appropriate to squid nerve at 6.3° C. have been inserted; the same values are used in all the computations referred to later unless otherwise mentioned. A rise of temperature is represented by increasing all the αs and βs with a Q_{10} of 3.0. The units are: membrane potential, mV deviation from resting potential in sea water (depolarization negative); membrane current density, μA/cm.^{2}; membrane conductances, mmho/sq. cm.; time, msec.; membrane capacity, μF/sq. cm.

$$I_{\mathrm{K}} = 36n^4(V - 12) \qquad (1)$$

$$\text{with} \quad dn/dt = \alpha_n(1 - n) - \beta_n n \qquad (2)$$

$$\alpha_n = 0.01(V + 10)\Big/\left(\exp\frac{V + 10}{10} - 1\right) \qquad (3)$$

$$\beta_n = 0.125 \exp\frac{V}{80} \qquad (4)$$

$$I_{\mathrm{Na}} = 120m^3h(V + 115) \tag{5}$$

$$\text{with} \quad dm/dt = \alpha_m(1 - m) - \beta_m m \tag{6}$$

$$\alpha_m = 0.1(V + 25)/\left(\exp\frac{V + 25}{10} - 1\right) \tag{7}$$

$$\beta_m = 4 \exp\frac{V}{18} \tag{8}$$

$$dh/dt = \alpha_h(1 - h) - \beta_h h \tag{9}$$

$$\alpha_h = 0.07 \exp\frac{V}{20} \tag{10}$$

$$\text{and} \quad \beta_h = 1/\left(\exp\frac{V + 30}{10} + 1\right) \tag{11}$$

In addition a "leak current"

$$I_L = 0.3(V + 10.613) \tag{12}$$

was assumed.

Membrane Action Potential

If a nerve fiber is stimulated simultaneously all over a considerable length (for example, by applying a shock to a long internal electrode), all points within that length will undergo an action potential simultaneously. There will be no differences of potential within the axis cylinder and therefore no longitudinal current; the resultant of the ionic currents through any area of membrane (together with any applied current I) must therefore go to charging or discharging the membrane capacity of the same area. This type of action potential is referred to as a "membrane" (as opposed to a propagated) action potential. The equation of such an action potential is therefore:

$$C_m dV/dt + I_K + I_{\mathrm{Na}} + I_L = I \tag{13}$$

C_m is taken as 1 μF/sq. cm.

Cole *et al.* (1955; Cole, 1958) were the first to put these equations on an electronic digital computer. They investigated chiefly the behavior of the equations near threshold, finding (Cole, 1958) that the response was not absolutely all-or-none if the strength of the stimulus was adjusted with extreme precision near the threshold. They also obtained repetitive firing in response to a constant current stimulus of well above threshold strength. In a certain range, the number of responses increased with current strength, but above a certain strength the train of impulses continued indefinitely.

More recently, the equations were set up on the electronic computers in the Mathematical Laboratory of the University of Cambridge, first, in 1956, on EDSAC I and in 1958 on the new and much faster EDSAC II. Standard subroutines were used for generating the functions required and for integrating the four simultaneous differential EQUATIONS 2, 6, 9, and 13; the integration

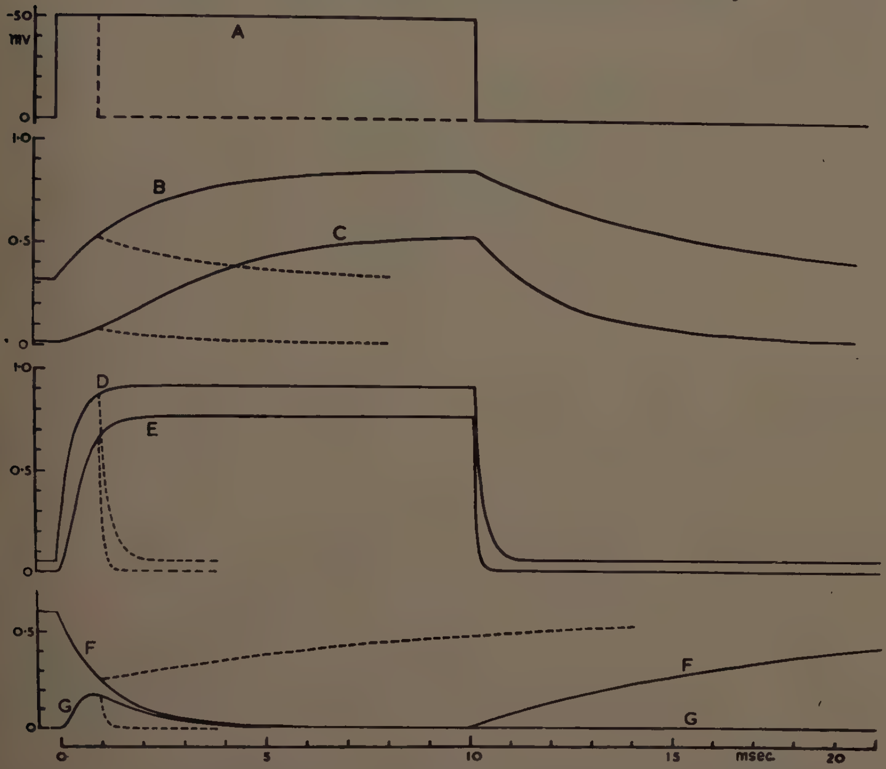

FIGURE 1. Diagram illustrating the manner in which the time course of the changes in permeability is fitted by the equations of Hodgkin and Huxley (1952*d*). The membrane potential is lowered by 50 mV from its resting level at zero time (curve A) and is restored after 10 msec. (solid lines) or 1 msec. (broken lines).

The curves at B show the corresponding changes in the quantity n given by EQUATIONS 2, 3, and 4, while C shows n^4, which was found to fit the observed time course of potassium conductance satisfactorily. Note that the curves at C rise with an S-shaped start, but fall in a nearly exponential manner.

Similarly, D shows m (EQUATIONS 6, 7, and 8), E shows m^3, F shows h (EQUATIONS 9, 10, and 11), and G shows m^3h. G fits the observed changes of sodium conductance; note (1) that it rises rapidly, but falls again during maintained depolarization, (2) that it can be reduced rapidly by repolarization (broken line), and (3) that on repolarization after a long depolarization there is no detectable rise in sodium conductance. The time scale is appropriate to 6.3° C.

was performed by a modification of the Runge-Kutta process. Both here and in Cole's work the original hand computations were satisfactorily confirmed.

Abolition of the Action Potential

It has been shown experimentally in several situations that an anodal shock of sufficient strength, applied during the rising or falling phase of an action potential, can restore the membrane potential fairly rapidly to its resting level, preventing the action potential from going through the subsequent parts of its normal time course. When this "abolition" response is obtained, it is found to bear a more or less all-or-none relationship to the strength of the anodal shock. The phenomenon has been shown in the early rising phase of a squid nerve action potential (Hodgkin *et al.*, 1949, Figure 7), in the plateau of the action potential of conducting tissue of the kid heart (Weidmann, 1951, Figure 6), and in the falling phase of the action potential of isolated myelinated nerve fibers (Tasaki, 1956; Lüttgau, 1956). It has been fairly clear intuitively that this behavior is explained by the reversibility of the change in Na permeability, the increase of which produced the rising phase of the spike as the fiber was originally depolarized, that is, it is due to a rapid decrease in the factor m (EQUATIONS 6, 7, and 8 and FIGURE 1, dotted part of curve G).

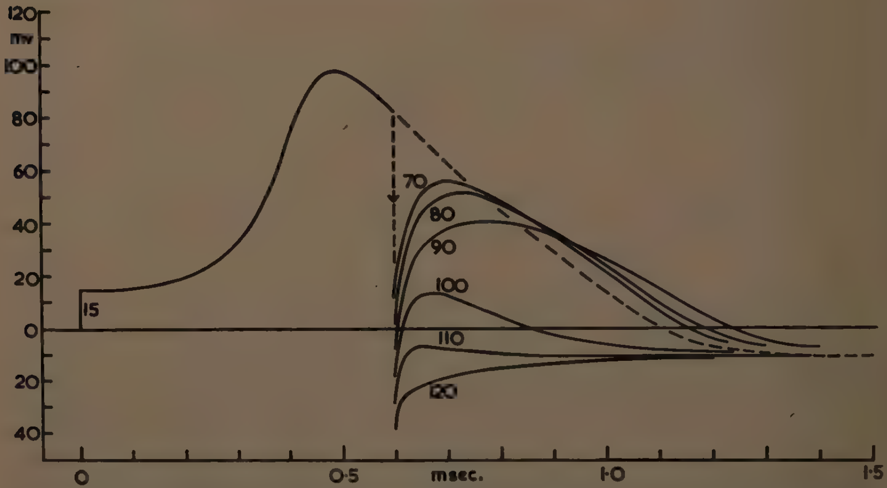

FIGURE 2. Calculated membrane responses of squid axon at 18.5° C. to a cathodal short pulse of 15 × 10^{-9} coulomb per sq. cm. at $t = 0$, followed by anodal short pulses of various strengths 0.6 msec. later. The broken line shows the time course of the membrane potential if the spike is not interrupted by an anodal pulse. The curves for anodal pulses of 70, 80, and 90 × 10^{-9} coulomb per sq. cm. return nearly to the time course of the uninterrupted spike, while those for 100, 110, and 120 × 10^{-9} coulomb per sq. cm. drop toward the level reached in the "positive phase" of the normal spike (that is, hyperpolarized by about 11 mV).

Some "action potentials" computed from the equations and showing the effect of an anodal displacement during the falling phase of the spike are shown in FIGURES 2 and 3. FIGURE 2 is computed for squid nerve at 18.5° C. It is seen that after a pulse of 90 × 10^{-9} coulomb/sq. cm. or less, the membrane potential returns nearly to the time course it would have had in a normal action potential, while with 100 × 10^{-9} coulomb/sq. cm. or more it drops quickly to the level reached in the positive phase. Since there are no comparable experimental results available on squid nerve, another set of responses was calculated with some of the parameters adjusted to make the behavior of the model nerve more like that of a node of Ranvier, for which experimental data are available in the papers of Tasaki (1956) and Lüttgau (1956). The changes made were suggested by the voltage-clamp data of Dodge and Frankenhaeuser (1958), and consisted in multiplying α_m and β_m by 4, that is, the speed of the process by which sodium permeability is turned on or off was increased fourfold. The results are shown in FIGURE 3. The shape of the action potential is more like that of a myelinated fiber, and the division between responses that return to the spike time course and those that drop to the level of the positive phase is more clear-cut than in FIGURE 2. The family of curves is in fact closely similar to that obtained experimentally by Tasaki (1956, Figure 7), showing that this representation of nerve activity gives a satisfactory explanation of the abolition phenomenon.

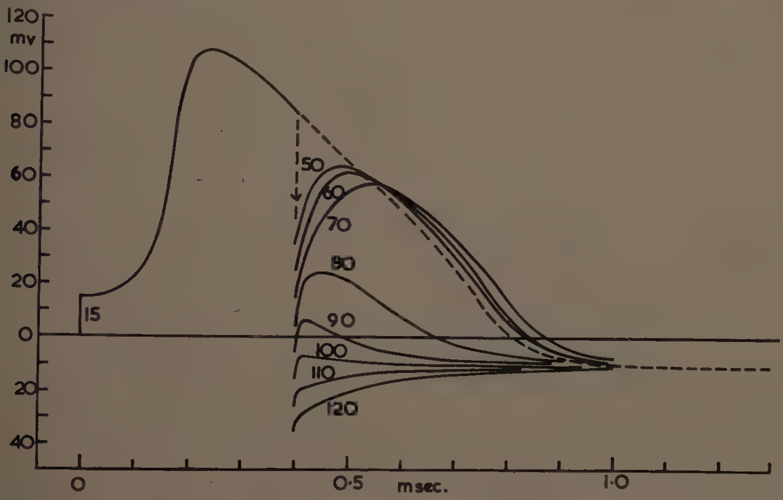

FIGURE 3. Calculated responses similar to those shown in FIGURE 2, except that the values used for α_m and β_m (EQUATIONS 7 and 8) were multiplied by 4. Pulse strengths are in units of 10^{-9} coulomb per sq. cm.

Reduced Calcium Concentration

The effects of changes in calcium concentration on the current-voltage relations in the membrane of the giant axon of the squid were investigated by Frankenhaeuser and Hodgkin (1957), using the voltage-clamp technique. They found that the main results could be "summarized by saying that the effects of a fivefold reduction of calcium on the system controlling Na and K permeability are similar to those of a depolarization of 10–15 mV." The formulation of the electric behavior of the membrane of the normal squid axon can therefore be modified to represent the behavior of an axon in solutions with increased or decreased Ca concentration simply by shifting all the functions for the αs and βs (which govern the permeability changes) along the voltage axis by an amount proportional to $\ln[\mathrm{Ca}]/[\mathrm{Ca}]_n$, where $[\mathrm{Ca}]_n$ is the normal calcium concentration, that is, in EQUATIONS 3, 4, 7, 10, and 11, V is replaced by $(V + \Delta V)$ where $\Delta V = k \ln[\mathrm{Ca}]/[\mathrm{Ca}]_n$. $[\mathrm{Ca}]_n$ (in a Mg-free solution) is taken as 44 mM; Frankenhaeuser and Hodgkin (1957) found that this concentration gave about the same stabilizing effect as sea water, which contains 11 mM Ca and 55 mM Mg; k has been chosen, slightly arbitrarily, so that a ΔV of $+15$ mV corresponds to a fivefold increase of [Ca].

The frequency and damping of oscillations of very small amplitude were also calculated on EDSAC I by the method described by Hodgkin and Huxley (1952*d*, page 538) for various values of the calcium concentration.

All calculations have been made for a temperature of 18.5° C.

Resting potential. Calculation of the equilibrium condition showed that a rise in [Ca] (positive ΔV) caused a decrease in resting potential, while reduction of [Ca] caused the resting potential to rise to a maximum at $\Delta V = -9$ mV ([Ca] = 16.8 mM). Further reduction of [Ca] caused the resting potential to fall to a minimum at $\Delta V = -22$ mV ([Ca] = 4.15 mM) and then to rise again, approaching the potassium potential. The curve is plotted in FIGURE 4. The general tendency for the resting potential to rise as [Ca] is lowered is due to the rising K permeability, while the reversed slope between $\Delta V = -9$ mV and $\Delta V = -22$ mV is due to the rise in steady-state Na permeability that occurs in that range.

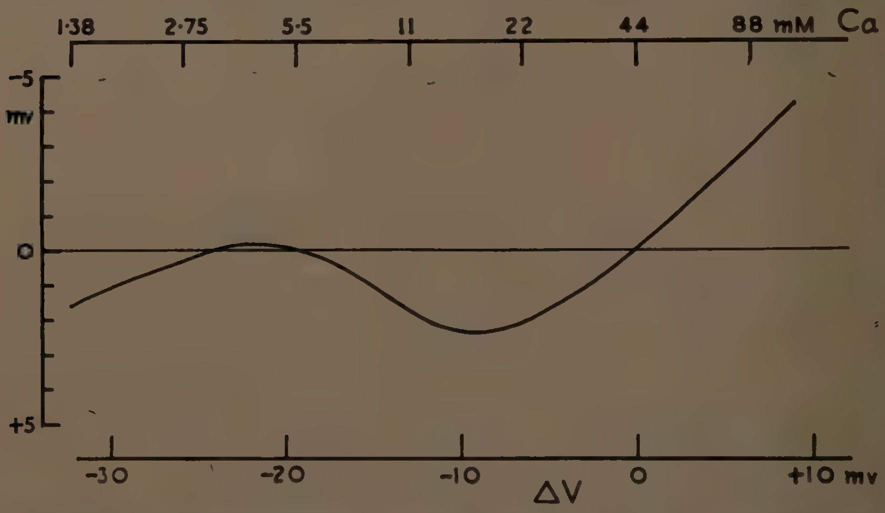

FIGURE 4. Calculated variation of resting potential with calcium concentration (depolarization upward).

Detailed experimental data on the squid axon are not available, but the results of Frankenhaeuser, Hodgkin, and Keynes (quoted by Frankenhaeuser and Hodgkin, 1957) are not inconsistent with this curve. They found a rise of 3 to 6 mV as [Ca] was reduced from 112 mM to 22 mM; at lower [Ca], "if the fibre was not firing spontaneously the resting potential was somewhat variable but was not usually displaced from its normal value by more than 5 mV."

Damping of oscillations. It was shown by Hodgkin and Huxley (1952*d*) that when the membrane potential of the model nerve (in normal [Ca]) is displaced by a small amount from its resting value, it returns with a heavily damped oscillation in much the same way as in the real squid axon. The frequency and damping of these oscillations (at 18.5° C.) have now been calculated for a range of values of ΔV, and the results are plotted in FIGURE 5. The frequency varies within the rather narrow range 110 to 150 cps unless [Ca] is lowered so far that the membrane is grossly unstable. The damping does not change much as [Ca] is raised, but gets progressively less as [Ca] is lowered

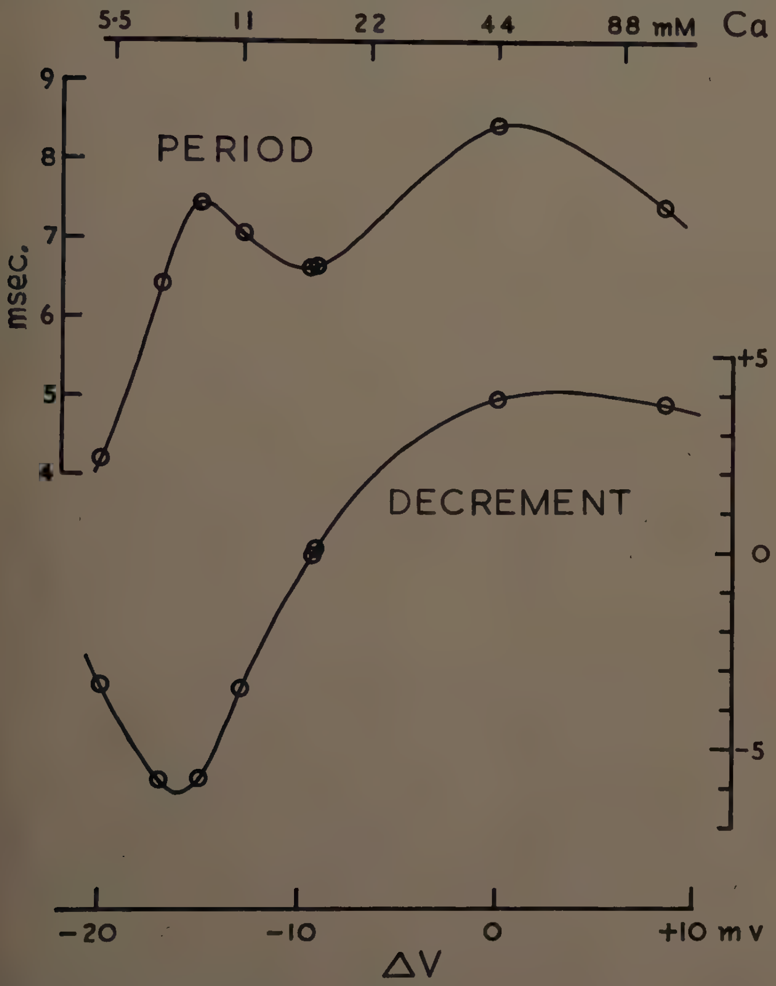

FIGURE 5. Period and decrement of oscillations of very small amplitude, calculated from the linear approximation to the equations of Hodgkin and Huxley (1952*d*), for 18.5° C. The unit of the decrement scale is the natural logarithm of the ratio of the height of each peak to that of its successor; for example, a value of -4 means that the amplitude increases by a factor of e^4 per cycle.

until at $\Delta V = -9.3$ mV ([Ca] = 16.2 mM) it reaches zero, and beyond that point it becomes negative, indicating that the amplitude of any small oscillation increases exponentially with time. The most rapid increase of amplitude occurs with $\Delta V = -16$ mV, where the amplitude rises by a factor of about 400 in each cycle.

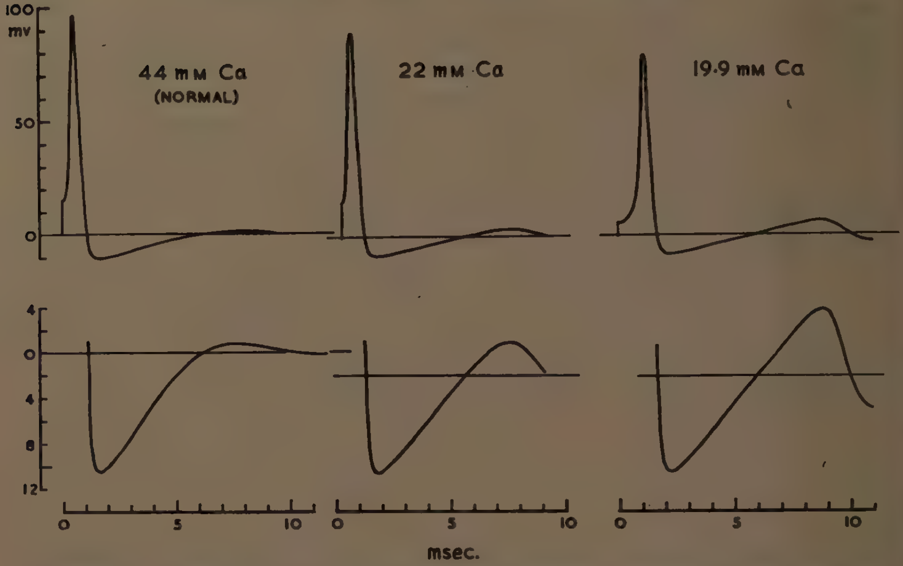

FIGURE 6. Changes in the time course of the calculated membrane action potential caused by moderate reduction of calcium concentration to below normal (44 mM). There is a marked increase in the oscillations that follow the spike, and a slight drop in spike amplitude. Calculated for squid nerve at 18.5° C.

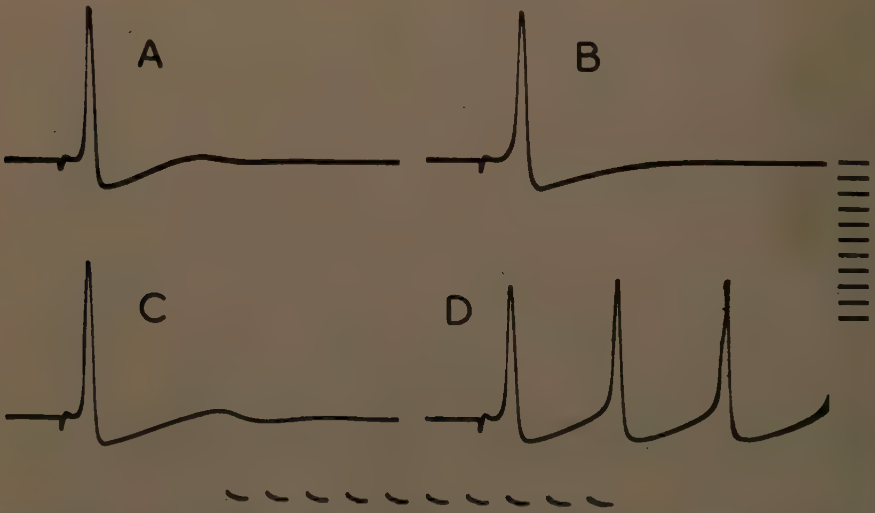

FIGURE 7. Propagated action potentials in a squid giant axon, recorded with an internal electrode by A. L. Hodgkin and B. Frankenhaeuser (unpublished data), showing a progressive increase in postspike oscillations as [Ca] is lowered until repetitive firing develops. A, in normal sea water (11 mM Ca + 55 mM Mg, equivalent to 44 mM Ca and 0 Mg); B, in a solution with 112 mM Ca and 0 Mg; C, in 22 mM Ca and 0 Mg; and D, in 4.4 mM Ca and 0 Mg. Temperature, 19° C.; calibrations, 10 mV and 2 msec.

There is no suggestion that there is any degree of decalcification at which the membrane potential would build up directly into a spike in response to a very small stimulus without first undergoing a series of increasing oscillations.

Generation of spikes. The spike generated by the model nerve (in normal [Ca]) in response to a sudden reduction of membrane potential was described by Hodgkin and Huxley (1952*d*). As ΔV is made progressively negative, representing decreasing Ca concentrations, the only changes that are noticeable at first are a decreased threshold and a slight increase in the damped oscillations that follow the spike (FIGURE 6). The corresponding alteration in the action potentials of a real squid axon is shown in FIGURE 7, which is composed of unpublished records taken by A. L. Hodgkin and B. Frankenhaeuser. It is seen that there is reasonably good agreement.

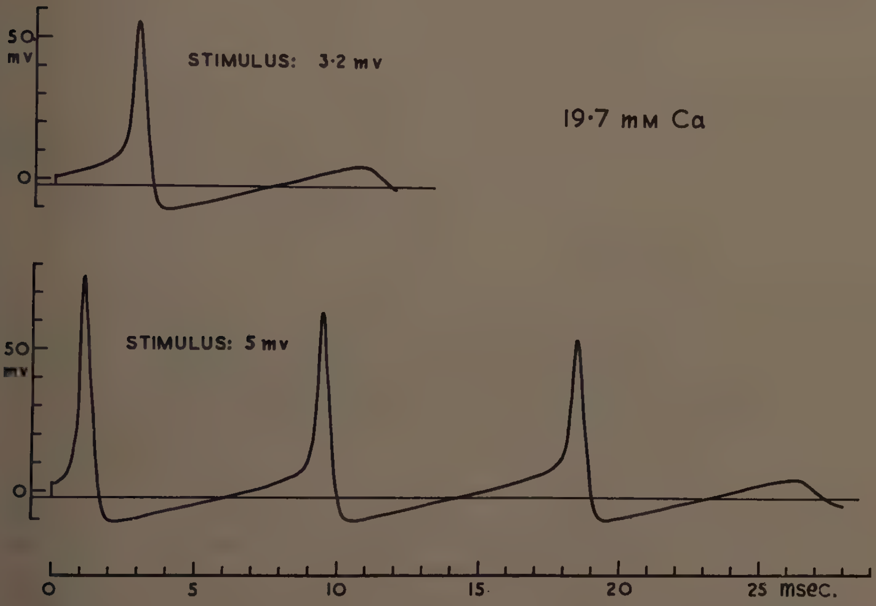

FIGURE 8. The first sign of a repetitive response induced by lowering the calcium concentration. *Top,* a just-threshold stimulus gives rise to a single spike. *Bottom,* a stimulus consisting of a short shock about 50 per cent greater than threshold gives rise to a train of 3 impulses of progressively decreasing size. Computed responses for squid nerve at 18.5° C.

The first qualitative change in the behavior of the model nerve is seen with ΔV between -7.4 and -7.5 mV ([Ca] between 19.7 and 19.9 mM), where the "nerve" responds to a just-threshold stimulus with a single spike, but gives a small number of spikes of slightly decreasing amplitude in response to a stimulus well above threshold (FIGURE 8). The range of [Ca] in which this type of response occurs is very narrow, and it is unlikely that it would be noticed in a real nerve unless it were specifically looked for; I am not aware of any case where such behavior in response to a short shock has been reported, but FIGURE 9 shows a record (by A. L. Hodgkin and R. D. Keynes) showing a group of 4 impulses in response to a long cathodal current stimulus, in a *Sepia* axon treated with a low-Ca solution (22 mM).

With further decalcification (ΔV from -7.5 to -8.5 mV approximately; [Ca] down to 17.7 mM), the threshold response consists of a series of spikes that is continued indefinitely, but a just subthreshold shock still causes a damped oscillation (FIGURE 10). It is conceivable that a single spike of much reduced size could be obtained by very precise adjustment of the stimulus strength to the threshold level, but this has not been attempted, and the corresponding result would not be observable experimentally since the random fluctuations of excitability are too large to allow a finite chance of hitting off the precise stimulus strength required.

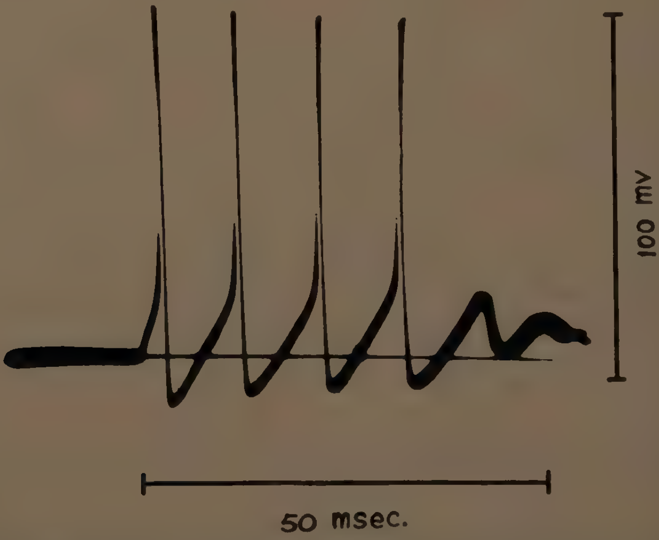

FIGURE 9. Short repetitive response to a constant-current stimulus in a *Sepia* axon in a solution with 22 mM Ca and 0 Mg. Internal recording at 17°.C.; calibrations, 100 mV and 50 msec. Unpublished record by A. L. Hodgkin and R. D. Keynes.

When ΔV is in the range from -8.5 to -9.3 mV ([Ca] from 17.7 to 16.2 mM), a cathodal stimulus may have 3 types of effect according to its strength. A weak shock causes only a damped oscillation of membrane potential, and a shock of sufficient size sets off directly a continuous train of spikes, but there is an intermediate range of strengths where an oscillation of increasing amplitude is established. This builds up until a spike is produced and, thereafter, a continuous train of spikes occurs. In this range, an anodal shock is very nearly as effective in stimulating the nerve as a cathodal one. Damped and increasing oscillations at the same [Ca], with cathodal and anodal shocks, are shown in FIGURE 11.

With ΔV below -9.3 mV ([Ca] $<$ 16.2 mM), the membrane potential is unstable even at its equilibrium ("resting") level, and any deviation produces oscillations that build up in amplitude until they develop into a train of spikes. Further decalcification down to $\Delta V = -21.5$ mV ([Ca] $=$ 4.4 mM) does not

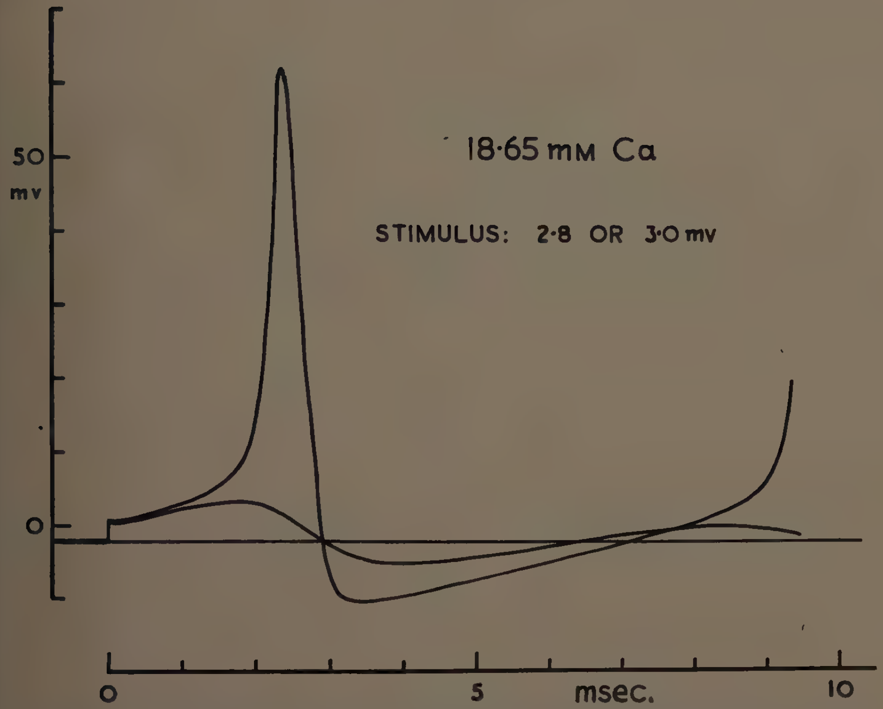

FIGURE 10. At a calcium concentration slightly lower than in FIGURE 8, a shock just above threshold produces a train of impulses of indefinite length, while a shock just below threshold causes a damped oscillation. Computed responses for squid nerve at 18.5° C.

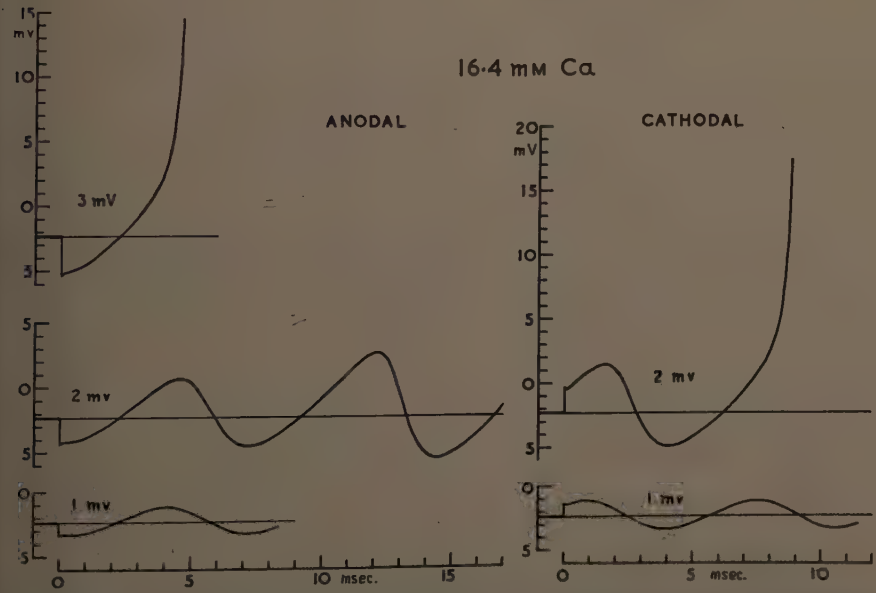

FIGURE 11. At 16.4 mM Ca a weak shock of either polarity initiates a weakly damped oscillation (bottom curves), a stronger one initiates an oscillation the amplitude of which increases until a train of spikes occurs (middle curves), and a still stronger one causes spikes to be produced without preliminary oscillations (*top left* for anodal shock; cathodal shock not computed for this [Ca], but similar to the threshold response in FIGURE 10). Computed responses for squid nerve at 18.5° C.

produce further qualitative changes, but the frequency of the discharge increases and the border line between "oscillations" and "trains of spikes" becomes progressively less definite (FIGURE 12). Beyond about $\Delta V = -23$ mV nothing but damped oscillations can be elicited, unless the nerve is pretreated with anodal polarization.

The progressive increase in frequency and eventual failure in a real squid axon are shown in FIGURE 13 (unpublished data of Hodgkin and Keynes);

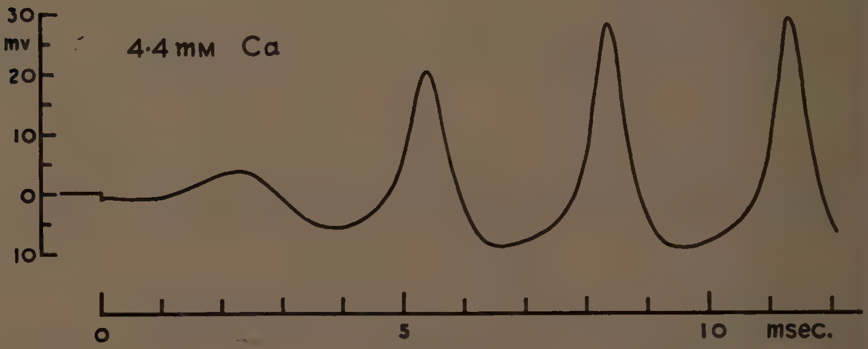

FIGURE 12. Computed response of squid nerve at 18.5° C. and calcium concentration only slightly above the minimum for spike responses. Stimulus: anodal shock displacing the membrane potential from its resting level (0.12 mV depolarized) to 1 mV hyperpolarized. Note the small amplitude of the spikes.

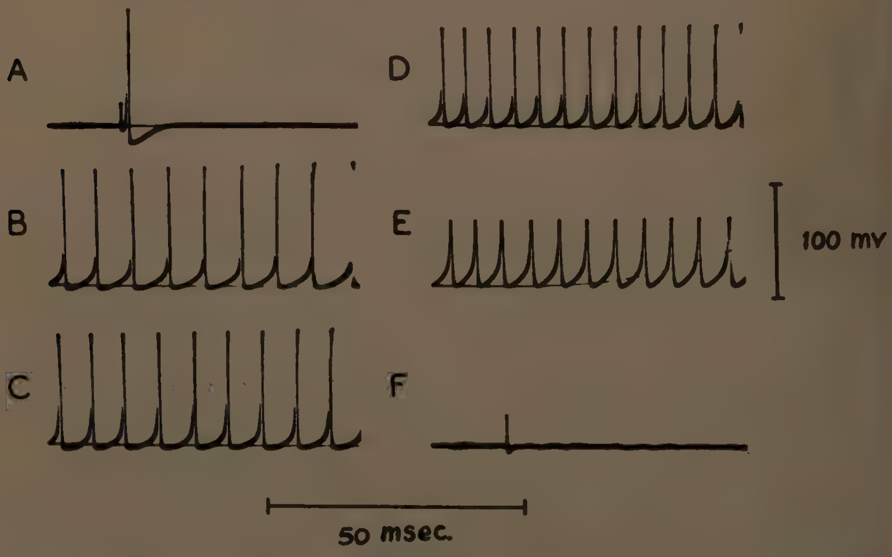

FIGURE 13. Responses of a squid giant axon, recorded with an internal electrode (propagated responses). A, in solution with 55 mM Ca, single spike in response to short shock; B, spontaneous firing 1 min., 25 sec. after change to 11-mM Ca solution; C, 2 min., 15 sec. after change to 4.4 mM Ca; D, 2 min., 40 sec. after change to Ca-free solution; E, 10 min., 30 sec. later; F, 3 min., 40 sec. later—no spontaneous activity and no response to a short shock. The axon again fired spontaneously when [Ca] was restored to 4.4 mM. Temperature, 17° C.; calibrations, 100 mV and 50 msec. (unpublished records by A. L. Hodgkin and R. D. Keynes).

it has also been found by Hodgkin and Frankenhaeuser (1957) that the squid axon becomes totally inexcitable in a Ca-free solution.

In the range where the decrement of subthreshold oscillations is near zero (either slightly positive or slightly negative), the frequency of the subthreshold oscillations is found to be very close to that of the train of spikes generated after threshold has been reached; this is illustrated in FIGURE 14 (the calcium concentration in this case is rather below the range at which the agreement is best), and in TABLE 1. This phenomenon has been observed by several authors (Arvanitaki, 1943*a*; Brink, Bronk, and Larrabee, 1946) and has been interpreted as indicating that the subthreshold process continues even when a train of spikes is being generated, and that each spike takes off from a corresponding wave in this underlying subthreshold process. The fact that a similar result is obtained in these computed responses shows, however, that this is not a necessary conclusion, since in this case it cannot be said that a subthreshold process continues while spikes are being produced; the situation is better described by saying that the system has two modes of oscillation (one subthreshold and the other above threshold), and that these have about the same frequency. To some extent this must be a coincidence, but it may be due partly to the fact that the spike itself takes up only a relatively small part of the superthreshold

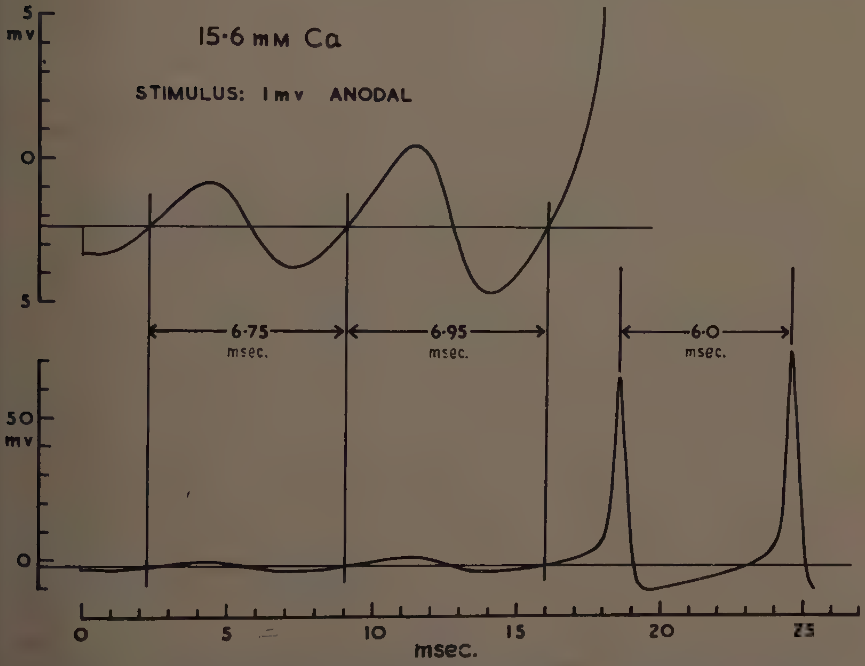

FIGURE 14. Computed response of squid nerve at 18.5° C., 15.6 mM Ca, plotted on 2 scales to show clearly the oscillations (*top*) and spikes (*bottom*). The interval between the spikes is not very different from the period of the subthreshold oscillation; the agreement is closer at a slightly higher calcium concentration where the subthreshold oscillations are barely regenerative (TABLE 1).

cycle, the rest of which is not grossly different from the corresponding parts of the subthreshold cycle.

The set of equations with which we began is thus seen to lead to responses that are in good general agreement with the activity of real cephalopod axons under more or less equivalent conditions of lowered calcium concentration. The comparisons have been made principally with Hodgkin's records because these were obtained on axons wholly immersed in the solutions with altered Ca concentrations, while most or all of the records in the literature are from axons to which the low Ca solutions had been applied only locally, the rest of the axon remaining in the normal solution. In this situation highly complicated interaction could evidently occur between a small but deeply decalcified region and nearby areas with normal excitability. It may be that this difference explains why some of the phenomena described by Arvanitaki (1943*a* and *b*) and others have not appeared in the computed responses (for example, maintained stable subthreshold oscillations and oscillations that fluctuate between the subthreshold and the superthreshold condition). It is conceivable, however, that alternation between subthreshold and spike responses might be obtained in the model system if the equations were extended to take account of after-potentials, or if the speeds of the delayed rectification (n) and inactivation (h) processes were chosen to be widely different.

TABLE 1
PERIOD (MILLISECONDS) OF OSCILLATIONS OF VARIOUS KINDS CALCULATED FOR SQUID AXON MEMBRANE AT 18.5° C. IN LOW-CA SOLUTIONS

ΔV mV	[Ca] mM	Very small amplitude	Damped oscillation	Increasing oscillation	Interval between spikes
0	44	8.4	≃8	—	—
−7.4	19.9	6.84	6.53	—	—
−7.5	19.7	6.82	—	—	8.3–9.0
−7.6	19.5	6.80	—	—	8.1
−8.0	18.65	6.75	6.60	—	7.3
−8.4	17.9	6.71	6.63	—	—
−8.8	17.1	6.67	—	7.5	6.5
−9.2	16.4	6.65	6.65	7.0–7.4	—
−9.7	15.6	6.63	—	6.7–7.0	6.0
−12.9	11	7.04	—	—	4.8
−21.5	4.4	—	—	3.2	3.0

The types of response that can be given by the model system at various degrees of decalcification are summarized diagrammatically in FIGURE 15. The shaded area represents (very roughly) the region that is difficult to investigate either experimentally or by numerical methods because the corresponding responses are obtained only if the amplitude is adjusted to threshold with extreme precision.

Propagated Responses

The computed responses thus far described have all been of the "membrane" type; that is, all potential changes have been assumed to be synchronous along the whole length of the fiber. If this assumption is not made, the problem becomes enormously more difficult, since EQUATION 13 is replaced by a partial differential equation in which distance along the axon, as well as time, appears as an independent variable. One special case, however, is still of just manageable proportions; namely, that of a disturbance traveling along the axon with constant form and velocity. These assumptions make the differentials with

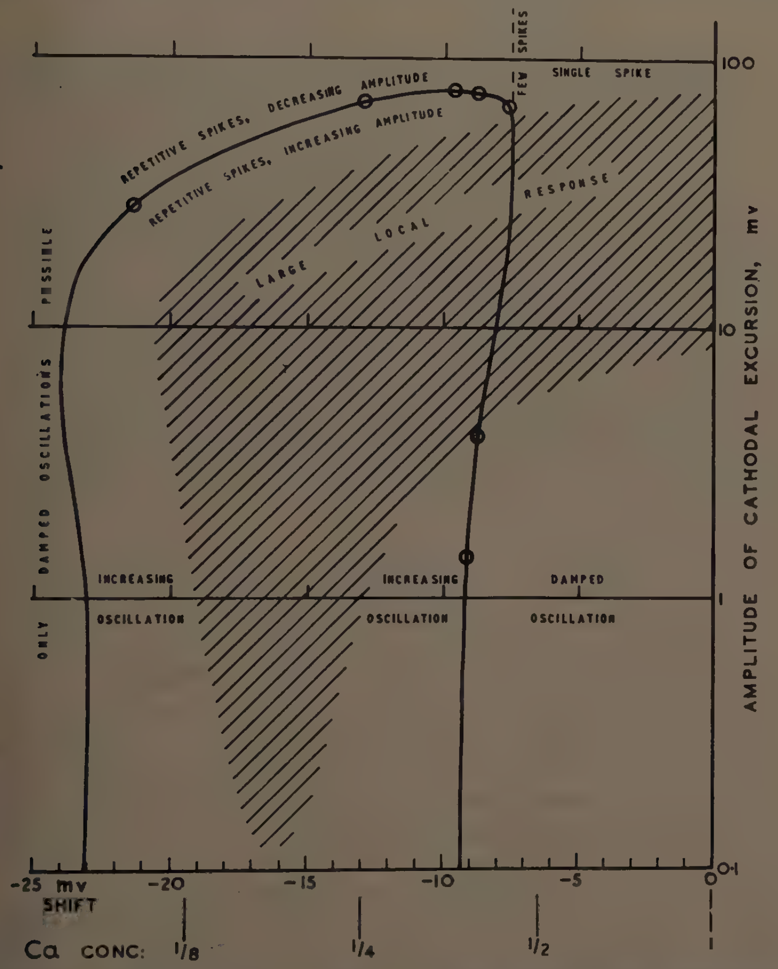

FIGURE 15. Diagram showing the types of response obtainable with various stimulus strengths at various calcium concentrations. All types of responses are regarded as oscillations; for example, a single action potential is an oscillation in which the first cycle is grossly unsymmetrical (spike followed by positive phase) and there is a very high degree of damping between the first and second cycles. The ordinate (logarithmic scale) is the amplitude of the swing in the cathodal direction; the abscissa is the degree of decalcification measured either as ΔV or as Ca concentration as a fraction of the normal value (44 mM). The continuous line is the locus of oscillations of zero decrement; its upper part, where it is concave downward, represents stable oscillations and its lower part represents unstable oscillations. The shaded area covers responses that can be only obtained by extremely precise adjustment of the stimulus in the vicinity of threshold. Above the shaded area the responses are spikes, and below they are subthreshold oscillations.

respect to distance and to time proportional to one another, so that the equation again can be written in terms of only one independent variable (either distance or time). Expressed in terms of time the equation is (Hodgkin and Huxley, 1952*d*, Equation 30):

$$\frac{a}{2R_2\theta^2}\frac{d^2V}{dt^2} = C_m\frac{dV}{dt} + I_K + I_{Na} + I_L \tag{14}$$

where a = radius of axon, R_2 = specific resistance of axoplasm, and θ = velocity. If a value of θ is chosen arbitrarily, and a solution is run off starting just on the depolarized side of the resting condition, it is found that V ultimately goes off toward either $+\infty$ or $-\infty$. These solutions correspond to disturbances traveling ahead of a moving anode or a moving cathode respectively, the velocity being smaller or greater than that of an unaided action potential. The velocity and time course of the normal spike must be found by a trial-and-error process: if V goes to $+\infty$ with a particular value of θ, the next run must be calculated with a larger value of θ and vice versa until a solution is obtained in which V remains finite for as long as is required. This procedure was very laborious for hand computation, and only a single solution was carried out for the 1952 paper. With EDSAC II, however, a solution giving the velocity to 6 decimal places and carrying the time course beyond the peak of the action potential (but not through the whole recovery phase) required only about 15 min. of machine time. The opportunity has therefore been taken of obtaining a series of solutions corresponding to different temperatures. The only parameters assumed to change with temperature have been the αs and βs; these have all been assumed to rise with a Q_{10} of 3.0 as the temperature is raised (Hodgkin *et al.*, 1952). If desired, the further effect of, for example, a change in the absolute magnitude of the conductances or in the resistance of the axoplasm can be calculated from these results by applying dimensional arguments; a method for doing this is described below.

The response calculated for a temperature of 18.5° C. agreed excellently with the hand-computed response, which was also made for this temperature.

The general features of the results can be seen from FIGURE 16, where they may also be compared with responses obtained experimentally at approximately the same temperatures by Hodgkin and Katz (1949*b*). The changes in amplitude, form, duration, and velocity (indicated roughly by conduction time) all agree satisfactorily with those in the experimental curves.

FIGURE 17 shows the variation of the calculated velocity with temperature; unfortunately there do not appear to be any experimental data available for comparison. The fact that the dotted part of the curve at the high-temperature end is turned back on itself, suggesting that 2 velocities are possible at each temperature, is not accidental. During the preliminary exploration of the temperature range, responses were calculated with very low velocities at temperatures of 31.5 to 32.6° C. (the values for the 2 right-hand points in FIGURE 17), and V was found to go toward $-\infty$, that is, the corresponding point on FIGURE 17 would be outside the line, which must therefore have curved back. The second velocity has not yet been calculated, but it presumably corresponds to a just-threshold disturbance traveling slowly along the nerve, evidently a highly unstable condition that might be impossible to observe experimentally.

The maximum temperature reached by the curve in FIGURE 17 is about 33° C. Admittedly, this occurs in the dotted portion, which is somewhat uncertain, but a trial point at 32.9° C., with a velocity corresponding to an ordinate of

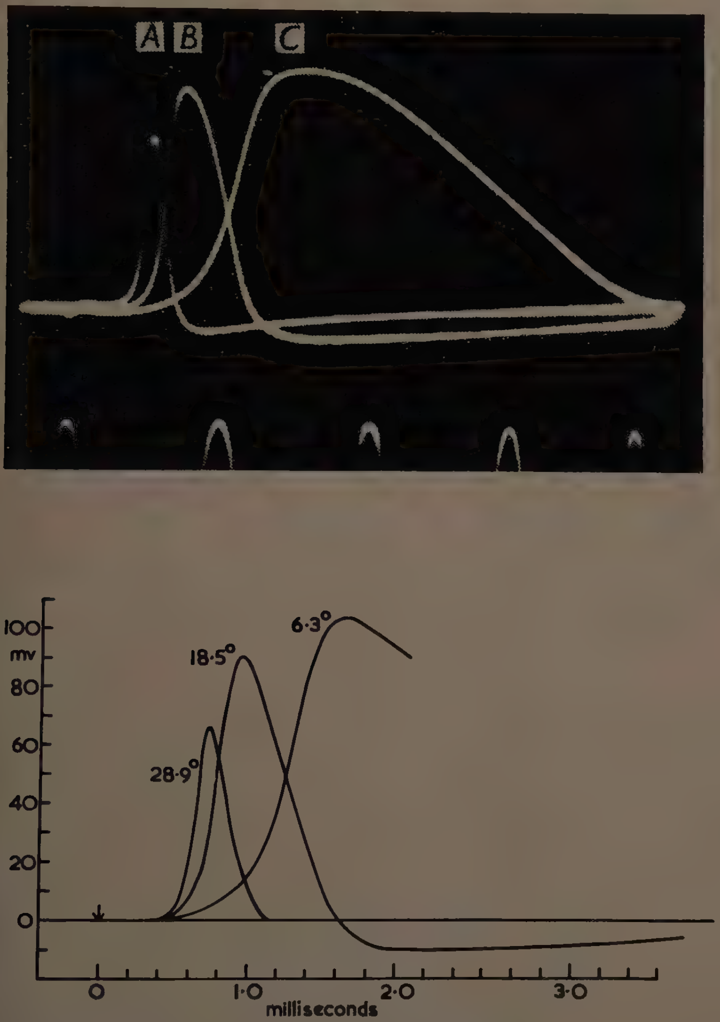

FIGURE 16. *Top:* superimposed records of action potentials at (A) 32.5° C., (B) 18.5° C. and (C) 5° C. The respective amplitudes are 74.5, 99, and 108.5 mV; time marks are 1 msec. (Figure 3 of Hodgkin and Katz, 1949*b*; reproduced by permission from *The Journal of Physiology*).

Bottom: propagated action potentials computed for the squid axon at 3 temperatures, allowing only for the effect of temperature in increasing the rates of change of the permeabilities. Each spike is drawn with a conduction time (measured at 30 per cent of the height of the spike) inversely proportional to its conduction velocity.

+0.05 on the graph made V go to $-\infty$, indicating that the curve keeps to the left of this point. The maximum temperature is thus fairly closely defined, and it represents a temperature above which the axon is incapable of propagating an action potential; thus a reversible condition of heat block should exist above this temperature. Exactly this phenomenon was found experimentally by Hodgkin and Katz (1949*b*), propagation being abolished when the temperature was raised above about 38° C. and restored when it was again brought below that temperature. Hodgkin and Katz suggested that the decrease in amplitude and disappearance of the action potential at high temperature were related to the fact that the Q_{10} of the rate of fall of the spike was greater than that of the rate of rise. This feature is also present in our computed responses, and is evidently one aspect of the reason for the block. Another way of looking at the matter is that, for the rate of rise of potential to increase as much as the rates of change of the permeabilities, the absolute values of the currents, and therefore of the conductances, would also have to be increased with the same Q_{10}, because the membrane capacity stays constant. Since we have assumed that the absolute values of the conductances are unaffected by temperature, the rising phase will be less accelerated and will tend to be overtaken by the permeability changes (inactivation of the sodium permeability and the rise of potassium permeability) that lead to recovery.

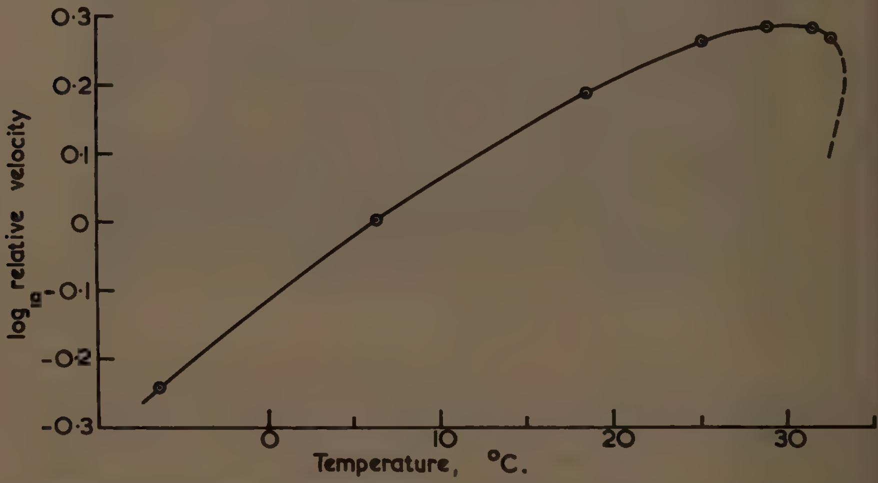

FIGURE 17. The effect of temperature on the velocity of the computed action potential allowing only for variation of rates of change of permeabilities ($Q_{10} = 3$). Velocity taken as unity at the standard temperature of 6.3° C.

FIGURES 18 and 19 show the variation with temperature of the amplitude and rate of rise of the computed spikes. In each case there is fair agreement with the experimental data from Hodgkin and Katz (1949*b*), but the computed spikes drop off in both respects in comparison with the experimental ones as the temperature is raised. It may be that the temperature coefficient of 3 is not quite correct, but this would not account fully for the discrepancy; more probably it is due to lack of allowance for an increase with temperature of the absolute values of the conductances. Hodgkin *et al.* (1952) found no clear evidence for such an increase and provisionally assumed that it did not exist; for the sake of uniformity the same assumption has been made here, but there is now evidence (Moore, 1958) that an increase does in fact occur.

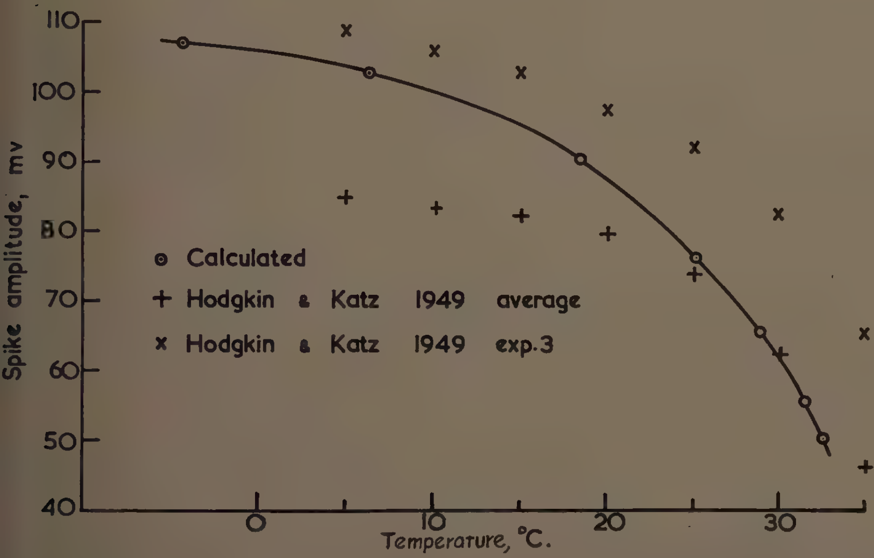

FIGURE 18. Amplitude of propagated spikes at various temperatures. Circles and curves are computed responses, allowing only for effect of temperature on rates of change of permeabilities; crosses, experimental results from Hodgkin and Katz (1949*b*).

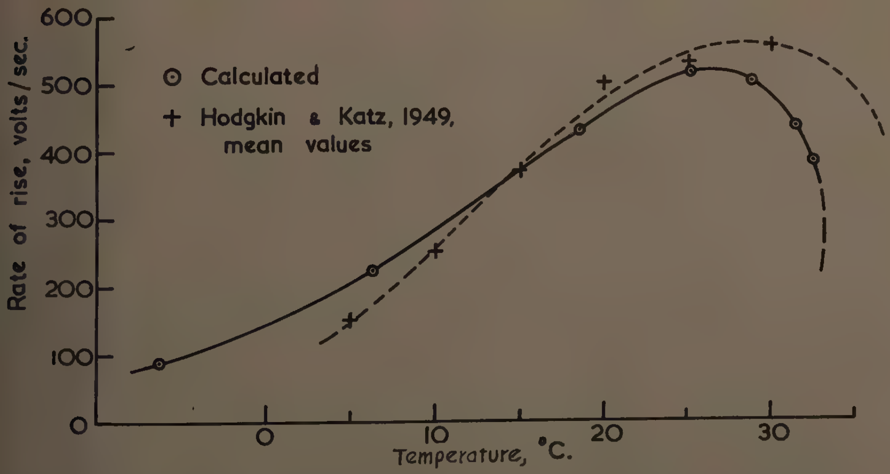

FIGURE 19. Rate of rise of propagated spikes at various temperatures. Full curve and circles are computed responses, as in FIGURE 18. The broken curve and crosses indicate experimental results from Hodgkin and Katz (1949*b*).

Dimensional Considerations on Conduction Velocity

It was indicated earlier that the results of the numerical computations presented here in terms of the effect of temperature can also be utilized to give the effect of other factors on the conduction velocity. The argument is as follows:

Equation 14 may be rewritten

$$\frac{d^2V}{dt^2} = K\left(\frac{dV}{dt} + \frac{I_i}{C_m}\right) \tag{15}$$

where I_i, the total ionic current, is a function of V and t (defined by equations 1 to 12), and

$$K = \frac{2R_2\theta^2C_m}{a} \tag{16}$$

Consider the effects of: (1) accelerating all permeability changes by a factor ϕ, (2) increasing membrane capacity by a factor γ, and (3) increasing the absolute values of all ionic currents by a factor η. With the symbols used by Hodgkin and Huxley (1952*d*), this means multiplying $\bar{g}_K$, $\bar{g}_{Na}$ and $\bar{g}_L$ by η.

Equation 15 then becomes

$$\frac{1}{K}\frac{d^2V}{dt^2} = \frac{dV}{dt} + \frac{\eta I_i(\phi t)}{\gamma C_m} \tag{17}$$

Now let $\tau = \phi t$, so that

$$\frac{\phi^2}{K}\frac{d^2V}{d\tau^2} = \phi\,\frac{dV}{d\tau} + \frac{\eta}{\gamma C_m}\,I_i(\tau) \tag{18a}$$

or

$$\frac{\phi}{K}\frac{d^2V}{d\tau^2} = \frac{dV}{d\tau} + \frac{\eta}{\gamma\phi}\,\frac{I_i(\tau)}{C_m} \tag{18b}$$

For any value of $\eta/\gamma\phi$ for which propagation is possible there will be a corresponding value of ϕ/K for which the solution does not go off to infinity, and therefore represents a normally conducted action potential. Hence

$$\frac{K}{\phi} = f\left(\frac{\eta}{\gamma\phi}\right) \tag{19a}$$

or

$$K = \phi f(\beta) \tag{19b}$$

where

$$\beta = \frac{\eta}{\gamma\phi}. \tag{19c}$$

From the definition of K (EQUATION 16) this becomes (remembering that the membrane capacity is now γC_m)

$$\theta^2 = \frac{a}{2R_2\gamma C_m}\,\phi f(\beta) \tag{20}$$

Each of the computed action potentials yields a value of $f(\beta)$ and the results are plotted in FIGURE 20 (on double logarithmic scales).

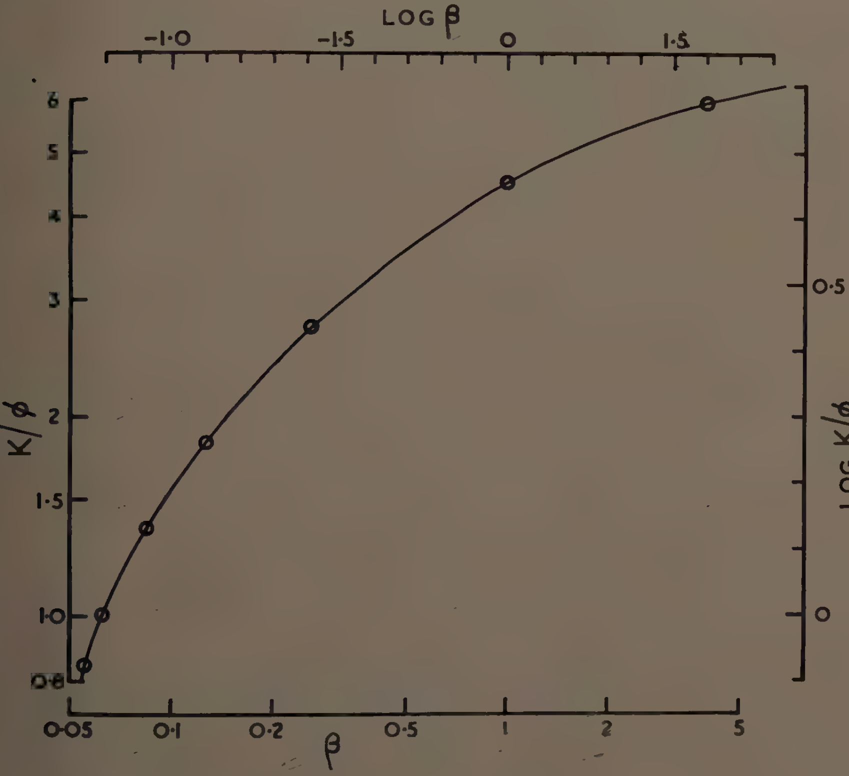

FIGURE 20. A plot of K/ϕ (= $f(\beta)$) against β on double logarithmic scales. Note the vertical scale is twice the horizontal.

As an example of the use of this graph, let us calculate the Q_{10} of conduction velocity between 10° C. and 20° C., assuming as before a Q_{10} of 3.0 for the rates of the permeability changes and in addition a Q_{10} of 1.5 for the absolute values of the conductances (Moore, 1958) and 1.3 for the conductivity of the axoplasm.

The "standard" temperature is 6.3° C., so that at 10° C., ϕ is $3^{3.7/10}$ (= 1.502) and η is $1.5^{3.7/10}$ (= 1.1618), giving β (= $(\eta/\gamma\phi)$) = 0.774, since the membrane capacity is assumed constant and therefore $\gamma = 1$. Similarly, at 20° C., $\beta = 0.387$. Reading off from FIGURE 20, we find $f(\beta) = 4.17$ at 10° C., and 3.24 at 20° C. From EQUATION 20 we see that the factors affected by temperature are $1/R_2$, ϕ, and $f(\beta)$. For these three quantities, the ratios of the 20° C. value to the 10° C. value are respectively 1.3, 3.0, and 3.24/4.17, so that

$$\frac{\theta_{20}}{\theta_{10}} = \sqrt{\frac{1.3 \times 3.0 \times 3.24}{4.17}} = 1.74$$

This is the Q_{10} that we set out to find, and it is reassuring that it is about the value generally quoted for nerve conduction velocity, although unfortunately there do not appear to be any values for the squid giant axon in the literature.

Another point of some interest is that, since the factors η, ϕ and γ all affect the velocity θ through the function $f(\beta)$ (see EQUATION 20), their effects must be interconnected, although they are not identical because ϕ and γ also appear as multiplying and dividing factors, respectively. If the logarithm of each side of EQUATION 20 is taken, and the result differentiated partially with respect to $\ln \eta$, $\ln \gamma$ and $\ln \phi$, the results are seen to be linked by the following relations:

$$\frac{\partial \ln \theta}{\partial \ln \phi} = \frac{1}{2} - \frac{\partial \ln \theta}{\partial \ln \eta} \tag{21}$$

$$\frac{\partial \ln \theta}{\partial \ln \gamma} = -\frac{1}{2} - \frac{\partial \ln \theta}{\partial \ln \eta} \tag{22}$$

$$\frac{\partial \ln \theta}{\partial \ln \gamma} = -1 + \frac{\partial \ln \theta}{\partial \ln \phi} \tag{23}$$

$(\partial \ln \theta)/(\partial \ln \eta)$ is equal to

$$\frac{1}{2}\frac{\partial \ln f(\beta)}{\partial \ln \beta}$$

or one quarter the slope of the curve in FIGURE 20 (since the vertical scale in the figure is twice the horizontal), and is always positive. EQUATION 22 thus shows, for example, that increasing the membrane capacity always causes a fall of velocity ($(\partial \ln \theta)/(\partial \ln \gamma)$ always negative), and that the effect is larger for small values of β than for large, since FIGURE 20 shows that $(\partial \ln \theta)/(\partial \ln \eta)$ is greatest for small β.

Conclusion

The computations that have been described extend greatly the range of phenomena that have been shown capable of explanation on the basis of the formulation of membrane properties proposed by Hodgkin and Huxley (1952*d*). The agreement with the observed behavior of real nerve fibers is gratifying, especially as the equations were used in exactly the same form, and (except for the case shown in FIGURE 3) with the same parameters, as were given in 1952, together with a single modification found by Frankenhaeuser and Hodgkin (1957) to represent the effect of changing the calcium concentration. As was recognized when the equations were first put forward, they do not cover processes such as after-potentials or the maintenance of the resting potential, but at present there does not seem to be any phenomenon involving only the spike processes themselves that cannot be brought within their scope. The values of the parameters and the actual form of the expressions used will undoubtedly require modification as better data become available, but there is no reason at present to think that the general formulation is wrong, and even the particular form in which the equations were cast in 1952 does not yet seem to have come to the end of its usefulness.

Acknowledgments

I thank A. L. Hodgkin for much valuable discussion, and Hodgkin, B. Frankenhaeuser, and R. D. Keynes for allowing me to reproduce the records shown in FIGURES 7, 9, and 13. I am also indebted to M. V. Wilkes, Director of the Mathematical Laboratory, Cambridge University, for permission to use EDSAC I and EDSAC II, and to D. J. Wheeler and other members of his staff for help in programming and running off the solutions.

Addendum

Since completing this paper I have confirmed on EDSAC II the existence of a solution of EQUATION 14 that represents a single (unstable) wave of depolarization followed by very small damped oscillations and traveling with a constant velocity much lower than that of the normal spike. At 6.3° C. the amplitude of this wave is about 13 mV, and its velocity is 0.225 times that of the spike, so that the corresponding point on FIGURE 17 would have an ordinate of −0.648. At 18.5° C. these values are 17.5 mV, 0.301, and −0.339, respectively. At each temperature still slower solutions exist that represent trains of 2 or more waves of depolarization that build up toward an amplitude of 30 to 40 mV.

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EXCITATORY AND INHIBITORY SYNAPTIC ACTION

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Electron microscopy has fully confirmed the neuron theory (Cajal, 1934) in that this technique has revealed the continuity of the surface membrane over the nerve cell, each nerve cell being structurally an independent unit. It has further shown that the functional connections (synapses) between nerve cells and between nerve and muscle occur across regions of very intimate contact, there being a gap (the synaptic cleft) of 150 to 400 Å between the respective presynaptic and postsynaptic membranes (De Robertis, 1956; Palay, 1956, 1958; Robertson, 1956, and personal communication).

The existence of two types of synaptic action, excitatory and inhibitory, was recognized long before intracellular recording gave information relative to the ways in which excitatory synaptic action evoked the discharge of impulses and in which inhibitory synaptic action was able to prevent this discharge. By degeneration experiments Szentagothai (1958) has recently produced the first evidence distinguishing between the actual synaptic structures responsible for these two opposed functional actions. When, by degeneration procedures, motoneurons were left with only the inhibitory connections from Renshaw cells, they were devoid of all synaptic knobs, but there still remained fine fibrils lacing over their surface. Complementarily, destruction of the inhibitory pathway to oculomotor neurons caused a degeneration only of a similar fine fibrillar structure. Hence it seems possible that inhibitory synapses are formed by the functional contacts made by these fine fibers, and that all of the conventional synaptic knobs are excitatory.

Synaptic excitatory action was first studied intracellularly by recording activity at the neuromuscular junction (Fatt and Katz, 1951), but this transmission lies outside the scope of the present treatment, which is especially concerned with those synaptic transmissions where there is antagonism between excitatory and inhibitory actions. Since the first intracellular studies on the motoneuron (Brock *et al.*, 1952), this dual synaptic action has been investigated in several other types of nerve-cell and nerve-muscle junction. It has therefore been thought appropriate to adopt a comparative approach to the problems of synaptic excitation and inhibition. Because of the much more extensive investigations on motoneurons, their responses will necessarily be treated more fully, but at each stage the relevant data for other synaptic mechanisms will be discussed in relation to the motoneuronal responses.

There have been two rival hypotheses, the electric and the chemical, for the manner in which an excitatory influence is transmitted across the synaptic contact between one nerve cell and the next. With the great majority of excitatory synapses it may now be taken as established that a nerve impulse causes a specific chemical transmitter to be released from the presynaptic terminals and thence to traverse the synaptic cleft and exert a depolarizing influence on the postsynaptic membrane; thus far, most of these transmitters in the central nervous system have not been identified. However, Furshpan and

Potter (1957) have shown that the synapse between the median giant fiber and the motor axon of crustacea operates according to the alternative hypothesis of electric transmission. One-way transmission is ensured by the rectification properties of the membrane lying between the presynaptic and postsynaptic elements. It appears that, if there is a synaptic cleft between these elements, it does not communicate with the extracellular space, which is an essential feature in the operation of a chemical transmitting synapse (Eccles, 1957; Eccles and Jaeger, 1958; Katz, 1958; Hagiwara and Tasaki, 1958), as may be seen below in the diagram of postsynaptic current flow (FIGURE 3*B*). The electric transmitting synapse is sharply distinguishable from the chemical transmitting synapse by the absence of any latent period between the presynaptic impulse and the consequent postsynaptic depolarization. Furthermore, besides the transynaptic effect of impulses, depolarization directly produced by application of current to the presynaptic element causes a corresponding depolarization of the postsynaptic element (Furshpan and Potter, 1957), which is in striking contrast to the absence of such transynaptic action with the chemical transmitting synapses (Kuffler, 1949; Bullock and Hagiwara, 1957; Hagiwara and Tasaki, 1958).

When there is virtually synchronous activation of synapses on a motoneuron, as in monosynaptic activation by the largest afferent fibers (Group Ia) from muscle, the membrane exhibits a depolarization with a characteristic time course that is independent of the number or origin of the impulses producing it (FIGURE 1*A* to *D*). The arrival of the presynaptic impulse is followed after a brief interval (about 0.3 msec.) by a rapidly developing depolarization with a maximum in 1 to 2 msec., followed by a slower phase of decay. With other types of nerve cell in the spinal cord a comparable time course has been observed for the excitatory postsynaptic potential (EPSP) (see Curtis *et al.*, 1958), but slower phases of rise and decline are seen in sympathetic ganglion cells (R. M. Eccles, 1955), and still slower in the ganglion cells of *Aplysia* (Tauc, 1958). On the other hand, the giant synapses of the stellate ganglion of *Loligo* produce EPSPs that have a faster time course than with motoneurons (Bullock and Hagiwara, 1957; Hagiwara and Tasaki, 1958).

Usually the discharge of an impulse is generated by a nerve cell when the EPSP attains a critical level (FIGURE 1*I*, *J*, and *K*), which is normally about 10 mV; that is, when a membrane potential of −70 mV is reduced to −60 mV (Brock *et al.*, 1952; Frank and Fuortes, 1956*b*; Coombs *et al.*, 1957). In attaining this critical value, the EPSP can sum directly with a depolarization produced by direct application of a current.

As shown in the electrically differentiated records of FIGURE 1*J* and *K*, the EPSP first initiates (at the double-headed arrow) an impulse in the initial segment of the motoneuron (axon hillock plus nonmedullated axon) and this IS spike, as it is called, in turn generates the SD spike in the soma-dendritic membrane of the motoneuron. These intracellular investigations of motoneurons show that there is no need to postulate that impulses are generated by some process of "transmitter potentiality" in addition to the EPSP. In other nerve cells the EPSP acts similarly in generating impulses (R. M. Eccles, 1955; Eccles, 1957; Bullock and Hagiwara, 1957; Curtis *et al.*, 1958; Phillips, 1958) with the exception that in the giant ganglion cells of *Aplysia* the critical level of depolarization is attained, as a rule, by means of partial spike responses that are superimposed on the EPSP (Tauc, 1958). A similar intermediate process of impulse generation occurs with chromatolyzed motoneurons (Eccles *et al.*, 1958).

In attempting to appreciate the factors governing the time course of the synaptically induced depolarization, it is first necessary to investigate the time course of potential changes produced across the postsynaptic membrane by application of brief rectangular current pulses. As indicated in FIGURE 1, by subtracting record *F* from *E*, and *H* from *G*, the potential change so produced rises with an approximately exponential time course to a steady state and, on cessation of the current, the decay is also approximately exponential. When nerve cells have no dendritic appendages, the electric time constants of the membrane may be taken as equivalent to these directly measured values. For example, with the giant ganglion cells of *Aplysia*, the time constants so deter-

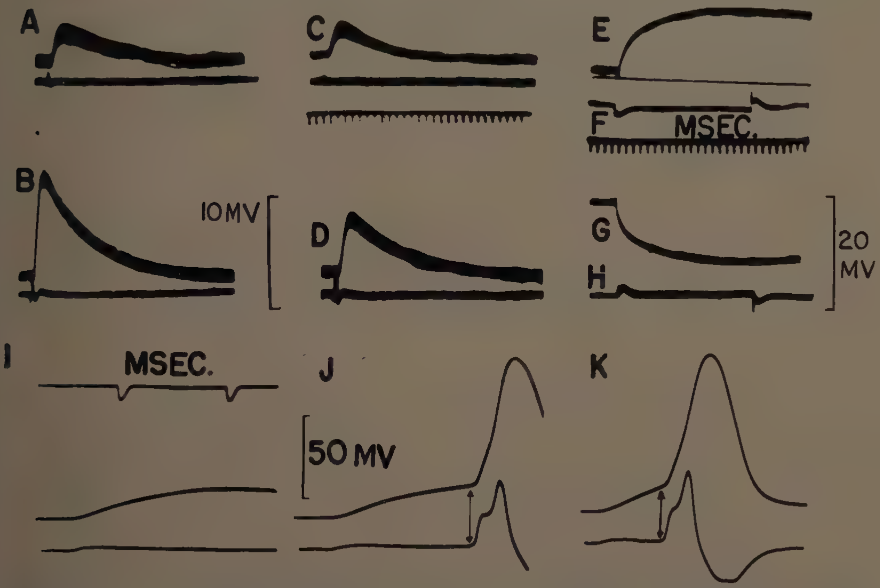

FIGURE 1. Intracellular recording through one barrel of a double microelectrode, which is inserted into a lateral gastrocnemius motoneuron with a resting membrane potential of −69 mV. EPSPs are produced by small and large Group Ia volleys from lateral (A and B) and medial gastrocnemius nerves (C and D). All records are formed by the superposition of about 40 faint traces. E and F show intracellular and just-extracellular records for the same motoneuron for a rectangular depolarizing pulse of 8×10^{-9} amp., while G and H are similar records for a hyperpolarizing pulse, the artifacts being largely balanced out by a compensatory circuit. Note the separate potential scales for the synaptic potentials and the pulse responses. Time scales are shown throughout in milliseconds (Coombs *et al.*, 1958). I, J, and K show, for a different gastrocnemius motoneuron and at a much faster sweep speed, the excitatory responses evoked monosynaptically by increasing size of afferent volley from I to K. In I, the EPSP is just below the threshold for generating an impulse, and in J it is just above it. The lower traces are the electrically differentiated records, the double-headed arrows indicating the onsets of the IS spikes (Coombs *et al.*, 1957). Reproduced by permission from *The Journal of Physiology.*

mined range from 10 to 80 msec. (Fessard and Tauc, 1956) and, with the supramedullary cells of the puffer fish, the time constant is 4 to 6 msec. (Hagiwara and Saito, 1957). In both of these cases the synaptically produced depolarization decays much more slowly (FIGURE 2; compare *B* with *C*, and *D* with *E*). Consequently, it must be assumed that the activated synapses have a residual depolarizing influence throughout the decaying phase of the EPSP, which has the effect of slowing the rate of its decline (see Tauc, 1958).

When, as with motoneurons, there are extensive dendritic branches from the cell body, the electric time constant of the membrane will be considerably longer than values derived from directly applied rectangular currents (see Rall, 1957). Using assumed values for the number and dimensions of the dendrites, an approximate allowance can be made for the dendritic distortion. On this basis the electric time constant of motoneurons has been derived from the time course of potential changes produced by directly applied currents (Coombs *et al.*, 1958). As with the approximately spherical neurons, the EPSP decays more slowly than would be expected for a purely passive decay of depolarization; here also the synaptic depolarizing action has a brief intense phase and a prolonged low residuum, which has been determined by analysis of the EPSP (continuous line) and is plotted as the broken line in FIGURE 3*A* (Curtis and Eccles, 1958). Two other independent lines of evidence also indicated the existence of the prolonged residuum of synaptic excitatory action.

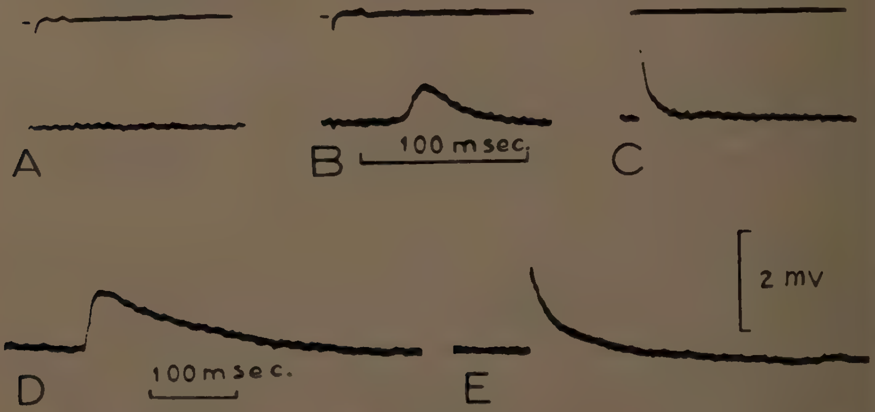

FIGURE 2. Intracellular records from ganglion cells of *Aplysia* with comparison between the time courses of EPSPs and potentials due to brief depolarizing current pulses. Afferent impulses were set up by stimulating electrodes and recorded intracellularly with a microelectrode, while the current pulse was applied through another intracellular microelectrode. In A, B, and C the ganglion cell was 70 μ in diameter, A being just below and B just above threshold for a single excitatory fiber, while C shows the potential produced by a very brief depolarizing pulse. D and E are similar to B and C, but for a large ganglion cell, 350 μ in diameter (Tauc, 1958). Reproduced by permission from *Rivista degli archivi italiani di biologia.*

This analytical determination of the time course of the synaptic depolarizing action has not yet been attempted with several types of central synapses, but a considerable duration is indicated by the repetitive discharges that are often evoked by a single afferent volley; as for example with the action of Group Ia afferent volleys from muscle on interneurons of the intermediate nucleus (Eccles *et al.*, 1956); with the action of cutaneous afferent volleys on dorsal horn neurons (McIntyre *et al.*, 1956; Eccles, 1958); and with the action of dorsal tract volleys on the cells of the cuneate nucleus (Amassian and de Vito, 1957). An extreme example is provided by the prolonged repetitive discharge evoked by a single excitatory volley in Renshaw cells (Eccles *et al.*, 1954; Frank and Fuortes, 1956*a*), the discharge being as long as two seconds when the destruction of the synaptic transmitter was prevented by a large dose of eserine.

On the other hand, with the giant synapse in the stellate ganglion of the squid, the decay of the EPSP is as rapid as would be expected from the time constant of the membrane (Bullock and Hagiwara, 1957), there being no appreciable residual transmitter action. The brevity of the transmitter action was very effectively demonstrated by the voltage clamp experiments of Hagiwara and Tasaki (1958), in which the postsynaptic current flow was measured directly. From the time course of this current and the measured time constant of the postsynaptic membrane it was possible to reconstruct satisfactorily the EPSP.

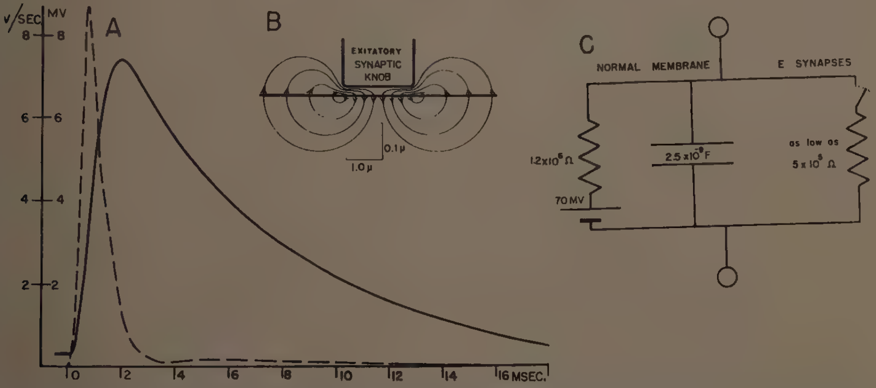

FIGURE 3. In A, the mean curve of the EPSP of FIGURE 1B is plotted as a continuous line and analyzed on the basis of the membrane time constant as derived from the records of FIGURE 1E to H to determine the time course (the broken line) of the postsynaptic currents that generate this potential change (Curtis and Eccles, 1958). The flow of this current relative to an activated synapse is shown diagrammatically in B for a synapse in which the vertical scale is exaggerated tenfold relative to the horizontal (see scales). In C there is a formal electric diagram of the membrane of a motoneuron; on the right side appears the circuit through the subsynaptic areas of the membrane that are activated in producing the monosynaptic EPSP. Maximum activation of these areas would be indicated symbolically by closing the switch.

As shown in FIGURE 3*B*, the activated synapses must depolarize the membrane by causing an inward flow of current in that part of the postsynaptic membrane acted on by the synaptic transmitter, with a corresponding outflow from the remainder of the membrane. By passing an extrinsic current across the neuronal membrane it has been possible even to reverse the resting membrane potential, the interior becoming positive to the exterior. When this occurs, the EPSP is also reversed in sign (FIGURE 4 at +9 and +34 mV), which indicates a reversal of the current flow: that is, an outward flow in the acti-

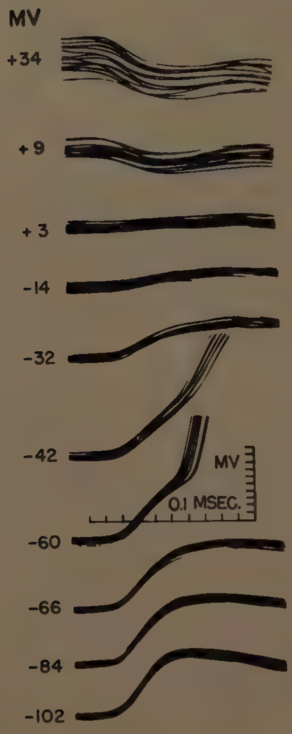

FIGURE 4. EPSPs set up in a biceps-semitendinosus motoneuron at various levels of membrane potential. Membrane potentials are indicated in millivolts to the left of each record as the potential of the interior of the cell with respect to the exterior. The resting potential was at −66 mV; the other potentials were obtained by the application of an extrinsic current through one barrel of a double microelectrode. In each record the decreasing internal negativity (depolarization from the resting level) or increasing internal positivity is upward. Each record is formed by superimposing 15 to 20 sweeps, the response occurring at the same relative time during the sweep. The scatter of individual sweeps in the records at greatest internal positivity is caused by fluctuations in the initial level of membrane potential. Spike potentials are evoked by the EPSP at membrane potentials of −42 mV and −60 mV (Coombs *et al.*, 1955*c*). Reproduced by permission from *The Journal of Physiology.*

vated membrane. When the EPSP is observed at various levels of membrane potential (FIGURE 4) it is found that the currents generating it are approximately proportional to the potential, which would be expected if the synaptic transmitter caused the receptive part of the postsynaptic membrane to become permeable to all mobile ions. Thus, the conditions causing the generation of the EPSP may be illustrated by the formal electric diagram of FIGURE 3*C*, where activation of the *E* synapses would cause the closing of the switch shown in the right element of the diagram.

A more precise study of this relationship has been made with the end-plate potential of amphibian muscle, and it has been found that the "null point" for reversal of the sign of the end-plate potential occurred when the interior of the membrane was about 15 mV negative to the exterior (del Castillo and Katz, 1954; Burke and Ginsborg, 1956; Katz, 1958), which is approximately the value for the calculated liquid junction potential between the interior of the fiber and its external environment (Nastuk and Hodgkin, 1950; Fatt and Katz, 1951). Thus it seems likely that, under the influence of the excitatory synaptic transmitter, the receptive patches of the postsynaptic membrane become permeable to all diffusible ions both inside and outside the membrane, which consequently move freely down their electrochemical gradients. On account of their relatively high concentration, Na^+, K^+, and Cl^- ions would be principally involved. At ordinary membrane potentials the inward current across the activated patches would be carried largely by Na^+ ions, which would be moving down a steep electrochemical gradient of over 100 mV across the membrane of about 50 Å thickness.

In a recent investigation Nastuk (this monograph) has found that the activated patches of the end-plate membrane are permeable to some substituted ammonium ions, but not to the very large ones. Similarly, the EPSP of motoneurons was essentially unchanged both in voltage and time course when, by electrophoretic injection, large changes were made in the ionic composition of motoneurons (compare FIGURE 8*C* and *D*, below, and Coombs *et al.*, 1955*c*). Thus, the general conclusion can be drawn that the activated membrane appears to behave as a sieve that allows the passage of all ions below a critical size.

Intracellular recordings from motoneurons revealed that inhibitory synaptic action produced a potential change (the IPSP) in the postsynaptic membrane that was in the reverse direction from the EPSP (Brock *et al.*, 1952). When both actions were due to virtually synchronized synaptic bombardments, the respective time courses appeared to be mirror images, but it has now been demonstrated (Curtis and Eccles, 1958), that the IPSP always has a more rapid decay than the EPSP (compare FIGURE 5*G* and *H*). As with the EPSP, the IPSP is altered in size, but not in time course, by grading the size of the inhibitory volley (FIGURE 5*A* to *F*), so it may be assumed that individual impulses generate an IPSP with the same time course. The decay of the IPSP follows an approximately exponential curve that has a time constant little if any slower than the membrane time constant (compare FIGURE 5*I* to *L*), so analysis reveals that the current generating the IPSP has a very rapid rise and decline with virtually no residual phase (broken line, FIGURE 6*A*). The brief reversal of current flow at 2 to 4 msec. is probably a result of the electrotonic distribution of the IPSP to the remote dendritic regions (Curtis and Eccles, 1958).

A hyperpolarizing potential with a similar time course has been observed for the IPSPs generated in other nerve cells either by single impulses or by virtually synchronous inhibitory volleys: in the cells of Clarke's column (Curtis *et al.*, 1958), in cells of the motor cortex (Branch and Martin, 1958), in the stretch-receptor cells of crustacean muscle (Kuffler and Eyzaguirre, 1955), and in the giant ganglion cells of *Aplysia* (Tauc, 1958). In FIGURE 7*A* and *B*, the effect of varying the membrane potential is shown to be remarkably similar for the IPSPs of cat motoneurons and *Aplysia* ganglion cells. Hyperpolarization of the membrane causes a reversal of the IPSP so that it is possible to find equilibrium potential at which the IPSP is zero. In FIGURE 7 this potential is approximately -80 mV for the cat motoneuron and -60 mV for the *Aplysia* ganglion cell; that is, the equilibrium potential is at about 10 mV more hyperpolarized than the normal resting potential of the membrane. When acted on by the inhibitory transmitter substance, the receptive patches of the postsynaptic membrane normally pass an outward current, as shown in FIGURE 6*B*, with the consequence that there is an addition to the charge on the remainder of the postsynaptic membrane, which thus is caused to exhibit a hyperpolarization—the IPSP. The conditions generating the IPSP are shown in the formal

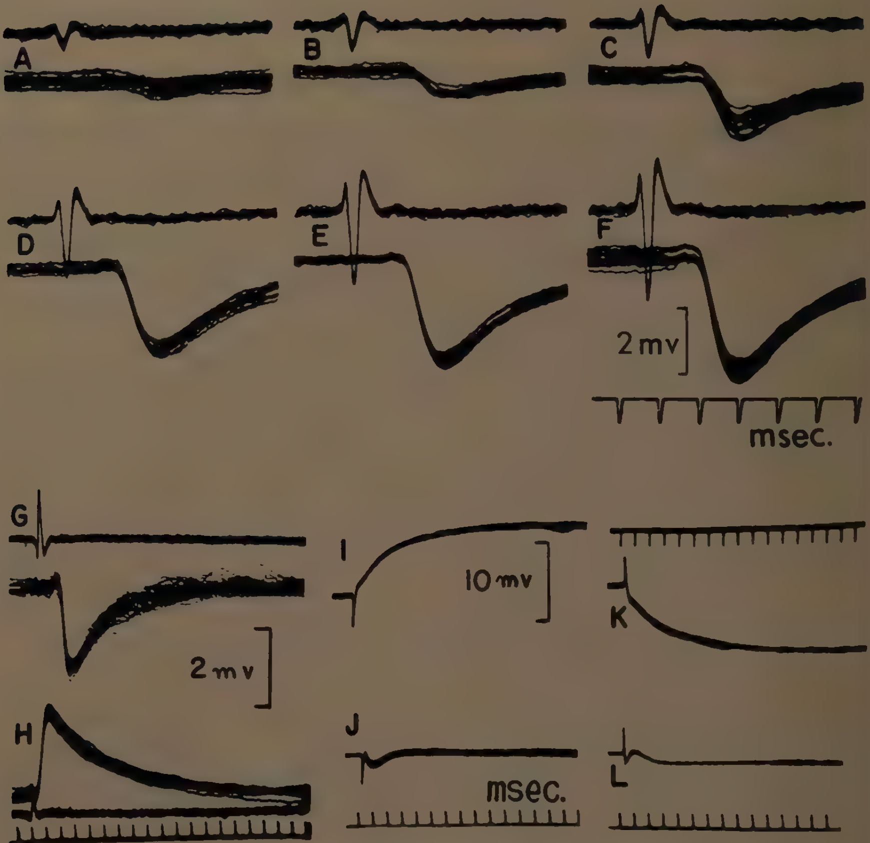

FIGURE 5. Lower records give intracellular responses (IPSPs) of a biceps-semitendinosus motoneuron to a quadriceps volley of progressively increasing size, as shown by the upper records, which are recorded from the L_6 dorsal root by a surface electrode, downward deflections signalling negativity. All records are formed by the superposition of about 40 faint traces (Eccles, 1958). G shows IPSPs similarly generated in another biceps-semitendinosus motoneuron, the monosynaptic EPSPs of this motoneuron being seen in H. I to L show changes in potential produced by an applied rectangular pulse of 12×10^{-9} amp. in the depolarizing and hyperpolarizing directions, just as in FIGURE 1E to H (Curtis and Eccles, 1958). Reproduced by permission from *The Journal of Physiology.*

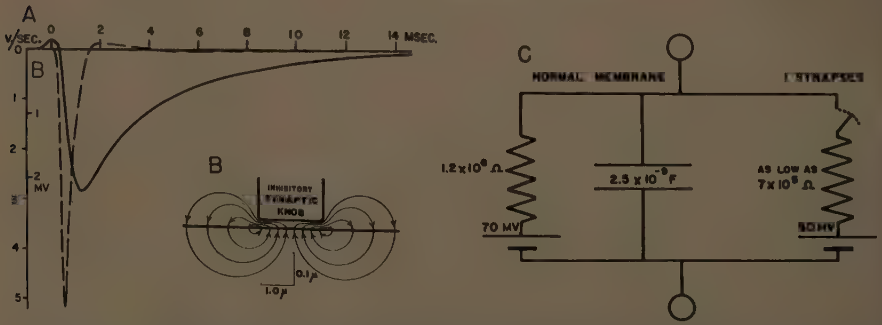

FIGURE 6. In A, the mean curve of the IPSP of FIGURE 5G is plotted as a continuous line and, on the basis of the electric time constant of the membrane determined from FIGURE 5I to L, it is analyzed as with FIGURE 3A to give the time course of the postsynaptic current generating it, as shown by the broken line. In B, the flow of these inhibitory postsynaptic currents is shown as in FIGURE 3B, while FIGURE 6C gives for inhibitory synaptic action the electric diagram corresponding to FIGURE 3C (Curtis and Eccles, 1958). Reproduced by permission from *The Journal of Physiology.*

electric diagram of FIGURE 6C, where activation of the I synapses would cause closure of the switch in the right element of the diagram.

With the crustacean stretch receptor the inhibitory potential is in the hyperpolarizing direction only when the cell is depolarized by the stretching of its associated muscle fiber. In full relaxation of this fiber the membrane potential

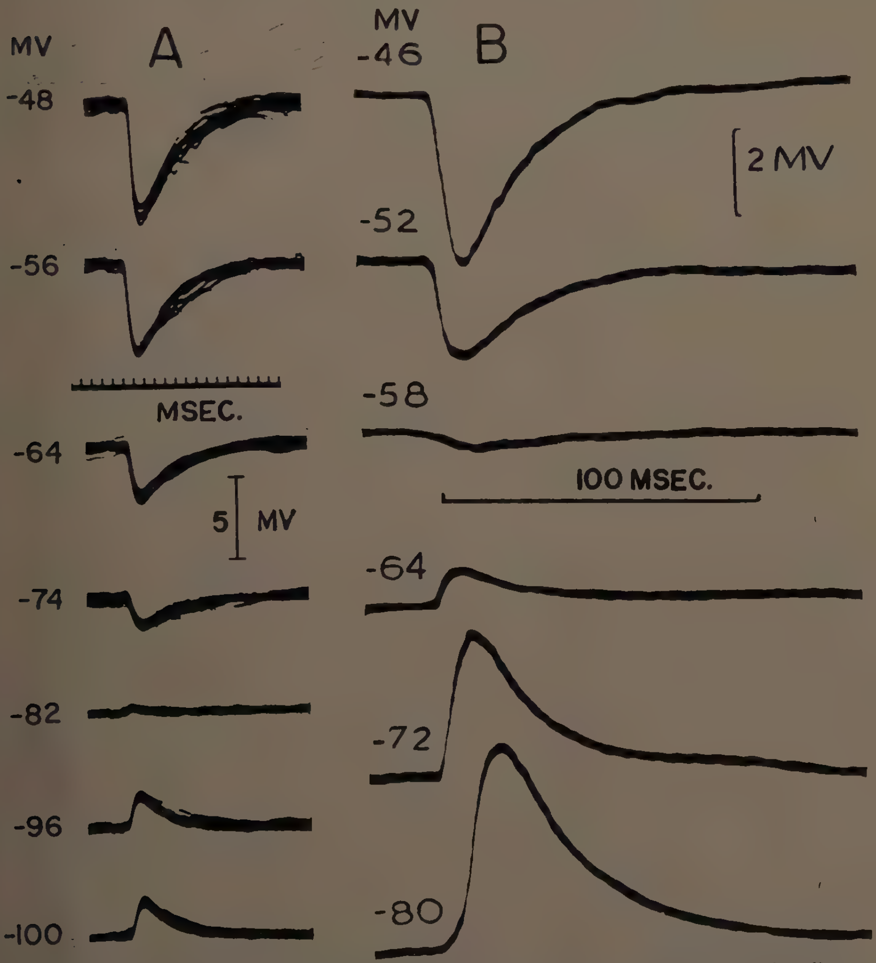

FIGURE 7. A shows the potentials recorded intracellularly from a biceps-semitendinosus motoneuron by means of a double-barrelled microelectrode. The records, formed by the superposition of about 40 faint traces, show the IPSPs set up by a quadriceps afferent volley. By means of a steady background current through the other barrel of the microelectrode, the membrane potential has been preset at the voltage indicated on each record, the resting membrane potential being −74 mV (Coombs *et al.*, 1955*b*). Reproduced by permission from *The Journal of Physiology.*

B shows the inhibitory potentials generated in a ganglion cell of *Aplysia* by a randomly fired single impulse in the same presynaptic fiber, showing, as in A, the effect of varying the membrane potential by means of a background current through another intracellular electrode, the membrane potential being indicated in millivolts on each trace (Tauc, 1958). Reproduced by permission from *Rivista degli archivi italiani di biologia.*

is approximately at the equilibrium potential for the IPSP (Kuffler and Edwards, 1958).

Two other inhibitory responses should be mentioned at this stage because they will be of interest when the ionic mechanisms are being considered. Inhibitory impulses cause virtually no change in the membrane potential of crustacean muscle fibers, but produce relatively large hyperpolarizing or depolarizing potentials of characteristic time course when the membrane potential is caused to be either lower or higher, respectively, than its normal level (Fatt and Katz, 1953; Boistel and Fatt, 1958). Thus, inhibitory responses of crustacean muscle fibers resemble those of the crustacean stretch receptor cell and differ from the IPSPs of the other nerve cells only in that the equilibrium potential of the IPSP is virtually the same as the resting membrane potential.

The inhibitory actions of vagal impulses on cardiac muscle fibers are usually complicated by the rhythmic process of impulse generation. Not only is there a suppression of the slow depolarizing process that leads to the initiation of impulses and a considerable decrease in the membrane resistance (Trautwein *et al.*, 1956), but usually there is also an increase in the membrane potential (Hoffman and Suckling, 1953; Hutter and Trautwein, 1955; del Castillo and Katz, 1955, 1957). It has been shown further that, if the membrane be sufficiently hyperpolarized, the inhibitory transmitter substance, acetylcholine, produces a depolarization instead of a hyperpolarization (Trautwein and Dudel, 1958). Thus, for vagal action on the heart there is an equilibrium potential for the inhibitory response that is on the hyperpolarizing side of the normal resting potential, being as much as 30 mV hyperpolarized for the resting dog atrium.

The action of inhibitory synapses in preventing the discharge of impulses by nerve cells is sufficiently explained by the depression produced in the depolarization of the EPSP. In part, this effect would arise on account of the actual hyperpolarization of the IPSP, but the depression of EPSP is much greater than would be expected from this action (Coombs *et al.*, 1955*d*). This additional effect is satisfactorily explained by the increase produced in the postsynaptic inhibitory current when the EPSP displaces the membrane potential farther from the equilibrium potential for the IPSP, as may be seen in FIGURE 7 for displacements caused by directly applied currents in either direction (see Eccles, 1957). The formal electric diagram of FIGURE 6*C* provides the basis for the explanation of this augmentation of the postsynaptic inhibitory currents. In this way it is possible to account for an effective inhibitory action against an excitatory depolarization even when the equilibrium potential for inhibition is virtually the same as the resting membrane potential, as occurs with the stretch receptor cells (Kuffler and Eyzaguirre, 1955) and the neuromuscular junctions of crustaceans (Fatt and Katz, 1953).

Under the favorable conditions provided by arrest of the rhythmic activity of the cardiac pacemaker, the excitatory and inhibitory actions of the sympathetic and vagus nerves on the heart have been found to be associated respectively with a depolarization and hyperpolarization (del Castillo and Katz, 1957), so it would seem that FIGURE 6*C* may also be applicable there. However, an exceptional situation arises with the depression of the EPSP by remote inhibitory action (Frank and Fuortes, 1957; R. M. Eccles and A. Lundberg, personal communication). Apparently, this inhibitory action is due to depression of the synaptic excitatory action and is not exerted on the postsynaptic excitatory response; that is, it is exerted farther upstream on the excitatory pathway than in all the other inhibitory actions we have been considering. An exceptional inhibitory mechanism also occurs with the catelectrotonically produced inhibition of Purkinje cells (Granit and Phillips, 1956).

The effects produced in the size and direction of the IPSP by varying the initial membrane potential correspond precisely to the changes that would be expected if the currents generating the IPSP were due to ions moving down their electrochemical gradients. As with the excitatory responses, these currents would be caused to flow by alterations in the ionic permeability that are produced in the specific inhibitory patches under the influence of the inhibitory transmitter substance. The nature of these permeability changes has been investigated by changing either the intracellular or the extracellular ionic composition.

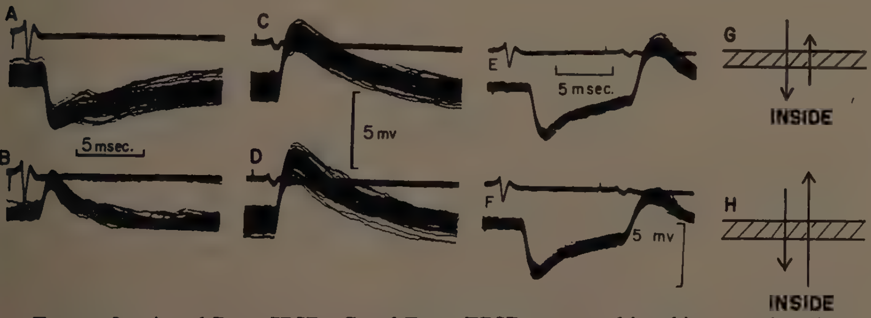

FIGURE 8. A and B are IPSPs, C and D are EPSPs generated in a biceps-semitendinosus motoneuron by afferent volleys as in FIGURE 5G and H, respectively. A and C were first recorded, then a hyperpolarizing current of 2 × 10^{-8} amp. was passed through the microelectrode, which had been filled with 3 M KCl. Note that the injection of chloride ions converted the IPSP from a hyperpolarizing (A) to a depolarizing response (B), while the EPSP was not appreciably changed (C and D). Passing a much stronger hyperpolarizing current (4 × 10^{-8} amp. for 90 sec.) through a microelectrode filled with 0.6 M K_2SO_4 caused no significant change (E to F) in either the IPSP or the later EPSP. G and H represent the assumed fluxes of chloride ions across the membrane before (G) and after (H) the injection of chloride ions, which is shown greatly increasing the efflux of chloride.

Thus, electrophoretic injection of chloride ions into motoneurons (Coombs *et al.*, 1955*b*) or into the cells of Clarke's column (Curtis *et al.*, 1958) causes the IPSP to change to a depolarizing response (FIGURE 8*A* and *B*). This inversion of the IPSP would be expected if the inhibitory transmitter acted by making the inhibitory patches highly permeable to chloride ions which, under such changed conditions, would exhibit a net flow outward down their electrochemical gradient, so depolarizing the membrane. For example, if the normal flux of chloride ions across activated inhibitory patches could be diagramed as in FIGURE 8*G*, an increased internal concentration of chloride would cause reversal of the net flux, as in FIGURE 8*H*, hyperpolarization giving place to depolarization. Parenthetically, it should be noted that it will later be argued that under normal conditions the hyperpolarizing IPSP is generated as shown in

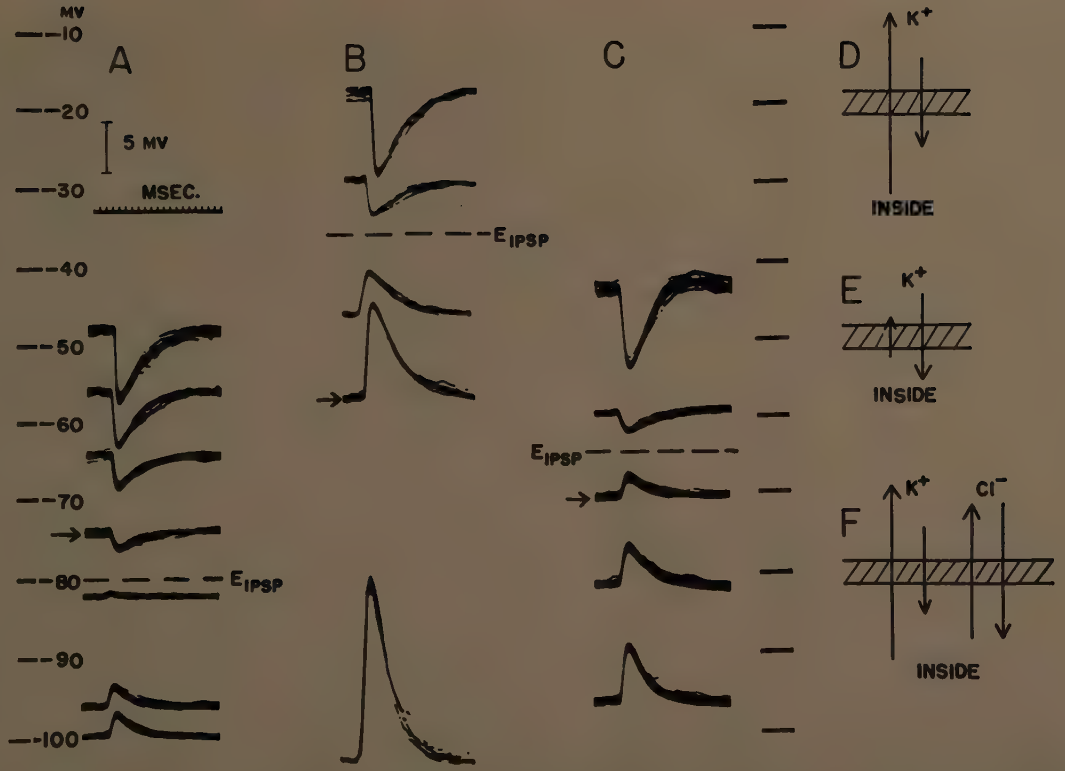

FIGURE 9. In A, the IPSPs of FIGURE 7A are shown arranged with their membrane potentials on the scale indicated by short horizontal lines to the left of A and right of C, and the equilibrium potential for the IPSP is shown by the broken line. B shows the situation after the passage of a depolarizing current of 5×10^{-8} amp. for 90 sec. through the microelectrode (filled with 0.6 M Na_2SO_4); the IPSPs are shown similarly arranged on the same potential scale, the E_{IPSP} being now −35 mV. C shows, on the same scale, the IPSPs obtained during partial recovery at 3 to 4 min. after the electrophoretic injection with the E_{IPSP} at −66 mV. D and E represent the postulated fluxes of K^+ ions in conditions A and B, respectively, while F gives the postulated fluxes of K^+ and Cl^- ions normally occurring across activated inhibitory postsynaptic membranes.

FIGURE 9*F*, where the chloride fluxes are equal, but the increased internal chloride would still cause a relative change in influx and outflux comparable with that in FIGURE 8*G* and *H*.

It was also found that electrophoretic injection of several other anions, Br^-, NO_3^-, and SCN^-, changed the IPSP to a depolarizing response, as in FIGURE 8*A* to *B*, while such anions as SO_4^{2-}, HPO_4^{2-}, $H_2PO_4^-$, CH_3COO^-, and HCO_3^- had no such effect (FIGURE 8*E* and *F*). Thus, one must assume that the inhibitory patches of the postsynaptic membrane become permeable to one group of anions, Cl^-, Br^-, NO_3^- and SCN^-, and not to the other group. In the hydrated state all members of the former group are much smaller than the members of the latter group; hence the simplest assumption is that the inhibitory transmitter has converted the specific inhibitory patches to a sievelike membrane whose pores exhibit a high degree of selectivity in respect of hydrated ion size.

The IPSP of motoneurons is also radically changed by the electrophoretic injection of cations other than potassium (Coombs *et al.*, 1955*b*). The effect of this injection is to cause a depletion of intracellular potassium, for the outward flux of potassium would be largely responsible for carrying the applied current across the cell membrane. Thus, intracellular injection of sodium or tetramethylammonium ions is very effective in depressing the equilibrium potential for the IPSP, causing it to fall even more than the resting potential; hence at the resting membrane potential the IPSP is converted from a hyperpolarizing to a depolarizing potential, as in the responses marked by arrows in FIGURE 9*A* and *B*. With intracellular sodium injection this effect is slowly reversible (compare with FIGURE 9*C*), presumably because the sodium is pumped out and potassium is regained. This effect of intracellular potassium depletion indicates that the inhibitory transmitter also increases the permeability of the inhibitory patches to potassium ions which, during the depletion, would exhibit a net inward flux (FIGURE 9*E*) in contrast to the original net outward flux (FIGURE 9*D*), with the consequence that the IPSP would become a depolarizing response.

Parenthetically, it should be mentioned that the current causing the electrophoretic injection of cations out of the electrode will, in addition, cause chloride ions to move from the external environment into the cell. As seen above, this would also tend to change the IPSP to a depolarizing response. However, this effect of inadvertent chloride injection can be controlled by injecting potassium ions electrophoretically. Consequently, there will not be any depletion of intracellular potassium and, correspondingly, it was found that the change in the IPSP was much smaller and more fleeting, as would be expected for the inadvertent chloride injection (Coombs *et al.*, 1955*b*).

Thus, it may be concluded that the inhibitory transmitter causes a brief increase in the permeability of the specific inhibitory patches of the motoneuron not only to the small anions, but also to potassium ions. At the same time a high degree of impermeability must be maintained to the larger cations such as sodium and tetramethylammonium; otherwise the observed potential changes would not be observed. The sievelike property of the membrane would obtain for the small cation, potassium, as well as for the small anions.

As a first approximation it appears that the membrane exhibits approximately the same permeability to potassium as to chloride. Investigations on the after-hyperpolarization of motoneurons have suggested that this potential change is solely due to potassium permeability (Coombs *et al.*, 1955*a*). The equilibrium potential for potassium derived therefrom is about −90 mV. With nerve and muscle cells it appears that the equilibrium potential for chloride is approximately at the normal resting membrane potential, that is, at about −70 mV for motoneurons. The observed equilibrium potential for the IPSP, about −80 mV, suggests that the currents generating it are due to a similar increase in both the chloride and potassium permeabilities, as indicated in FIGURES 9*F* and 10*A*. Thus, for motoneurons the conductance of the I synaptic element in FIGURE 6*C* would be shared by the approximately equal permeabilities to K^+ and Cl^- ions.

Tauc (1958) has likewise found that the equilibrium potential for the IPSP of the giant ganglion cells of *Aplysia* lies between the equilibrium potential for potassium and the resting membrane potential. Hence he has concluded that, as with the motoneuron, the IPSP is generated by an increased permeability to both potassium and chloride ions (FIGURE 10*A*), and that a high impermeability is maintained for sodium ions.

There has been a preliminary report (Edwards and Kuffler, 1957) that the movement of chloride ions is an important factor in producing the IPSP of the crustacean stretch receptor cell. More recent work (Kuffler and Edwards, 1958) has thrown doubt on this interpretation, and in addition has revealed that the potential is largely, if not entirely, generated by the movement of potassium ions. Thus, removal of potassium from the extracellular fluid causes the expected increase in the resting membrane potential, but the equilibrium potential for the IPSP is even more increased. Necessarily, a small concentration of potassium will be maintained in the just extracellular position, so this high equilibrium potential for the IPSP could be taken to indicate that the flux of potassium ions alone is significant in producing the IPSP. Certainly, the flux of chloride ions would play a subordinate role at most, and the inhibitory synaptic diagram may be drawn provisionally as in FIGURE 10*B*.

The ionic movements responsible for vagal inhibition of the heart have been studied chiefly by applying the inhibitory transmitter, acetylcholine, or one of its analogues. In this way it has been shown that inhibition is associated with a large increase in the potassium permeability of the cardiac muscle fibers (Burgen and Terroux, 1953; Trautwein and Dudel, 1958). Correspondingly, radiotracer investigations have shown that acetylcholine causes a large increase in the potassium permeability of the sinus venosus of the frog heart (Harris and Hutter, 1956). It appears that the observed potassium permeability is sufficient to account for the potential changes and for the increase in the electric conductance of the membrane (Trautwein *et al.*, 1956). There can be little if any increase in chloride permeability, so again FIGURE 10*B* gives the inhibitory diagram for vagal action on the heart.

In contrast to these two preceding examples, the inhibitory action on crustacean muscle fibers appears to be produced almost entirely by an increase in chloride permeability (Boistel and Fatt, 1958). The equilibrium potential for the IPSP was almost identical with the normal membrane potential. When the external chloride was replaced by large organic anions, the resting membrane potential fell by 10 to 20 mV, but the equilibrium potential for the IPSP fell by more than 30 mV, the hyperpolarizing response being very small even when the membrane was heavily depolarized by an applied current. Thus, it would appear that a hyperpolarization reaction is dependent on the inward movement of chloride, outward potassium movements making a negligible contribution even under very favorable conditions. This was shown further by the very small changes in the IPSP produced either by removing external potassium or by raising it to several times the normal value. The inhibitory diagram for crustacean muscle would be given by FIGURE 10C.

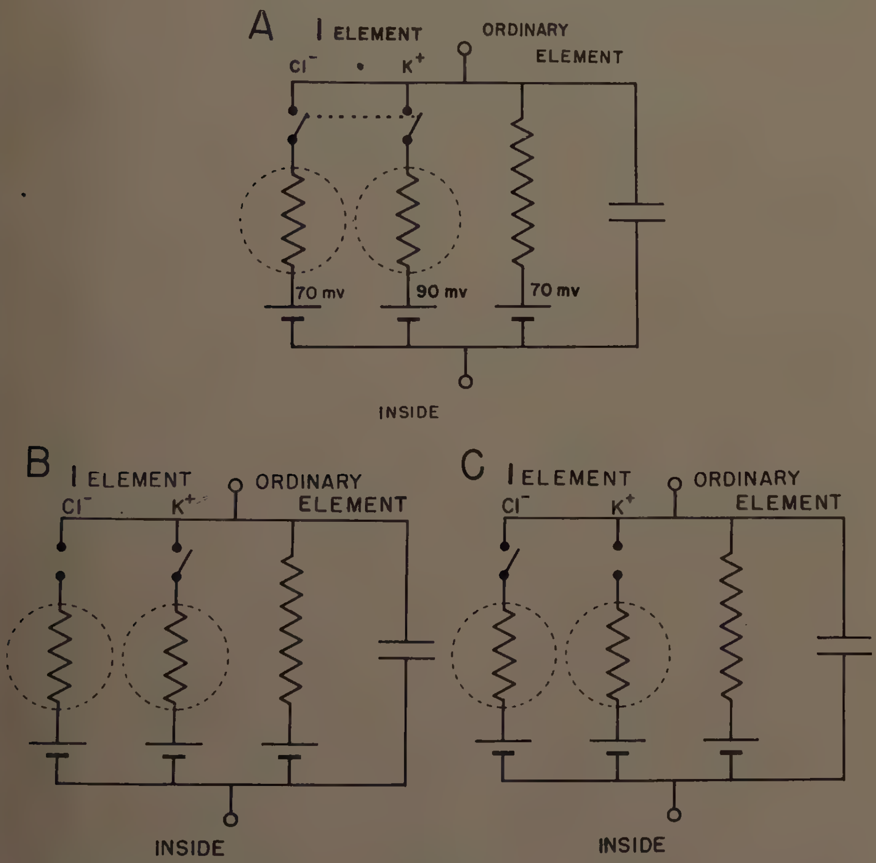

FIGURE 10. A is a diagrammatic representation of the electric properties of an ordinary element of the neuronal membrane and of an inhibitory element with K^+ and Cl^- ion conductances in parallel. The voltages are the mean values for a motoneuron, and would be somewhat lower for ganglion cells of *Aplysia*. B shows an inhibitory diagram for inhibitory elements in crustacean stretch-receptor cells and cardiac muscle. C shows an inhibitory diagram for inhibitory elements in crustacean muscle.

Since all inhibitory synaptic actions are associated with increases in ionic permeability, it would be expected that there would be an associated increase in membrane conductance. It is not possible to give a value for the change in conductance of the specific inhibitory patches, but this can be done for the change in conductance for these patches, together with the postsynaptic membrane effectively in parallel with them. For example, this conductance is at least doubled for the crustacean stretch receptor cell, and often the change is much greater (Kuffler and Eyzaguirre, 1955). The conductance increase may be almost doubled with the motoneuron (Eccles, 1957), and single inhibitory impulses produce an increase of up to 40 per cent in the membrane conductance of the giant ganglion cells of *Aplysia*. With crustacean muscle the increases are smaller, usually about 10 to 20 per cent (Fatt and Katz, 1953; Boistel and Fatt, 1958).

From the foregoing examples it would seem that the very diverse ionic mechanisms of FIGURE 10*A*, *B*, and *C* are employed in producing inhibitory effects. However, as Boistel and Fatt (1958) point out, it is possible to account for them all by a very simple addition to the postulate of a sievelike membrane that was developed to explain the contribution of various ions to the inhibitory responses of motoneurons (Coombs *et al.*, 1955*b*). According to this hypothesis, the inhibitory transmitter caused the specific patches of the postsynaptic membrane to become permeable to both anions and cations below a critical size (FIGURE 10*A*). This postulate will also account for the inhibitory responses of the giant ganglion cells of *Aplysia* (Tauc, 1958). If the pores of the postsynaptic membrane had the same dimensions but were negatively charged, they would become selectively permeable to cations, as was observed for potassium with crustacean stretch-receptor cells and with cardiac muscle fibers (FIGURE 10*B*). Potassium is the only small cation investigated to date. If, on the other hand, the pores were positively charged, they would exhibit the selective chloride permeability found for the crustacean muscle fibers (FIGURE 10*C*). It would be expected that such other anions as bromide, nitrate, and thiocyanate could be substituted for chloride in the inhibitory responses of crustacean muscle, but apparently this has not been tested as yet.

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IS THE RESTING POTENTIAL OF RANVIER NODES A POTASSIUM POTENTIAL?*

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The membrane theory of Julius Bernstein (1902) assumes that the potential difference (PD) existing between the interior of muscle or nerve cells and the extracellular fluid is caused by the concentration gradient of potassium if the membrane separating the two media is selectively permeable to potassium rather than to chloride and sodium ions. Owing to the high intracellular potassium concentration, the resting membrane of these excitable structures would then be continuously polarized, and the inside would be negative with respect to the extracellular fluid. This would agree with the observation that injured parts of nerve or muscle are negative compared to uninjured regions of the same structure. Bernstein based this theory on thermodynamics, proving that within reasonable limits the injury potential of nerve and muscle varied in proportion to the absolute temperature, thus presenting the characteristics of a concentration cell. Ever since his report efforts have been made to prove or to disprove Bernstein's hypothesis. The most striking evidence in favor of it was obtained by changing the extracellular potassium concentration $[K]_e$ and finding a linear relationship between membrane potential (V_m) and log $[K]_e$, at least if $[K]_e$ was varied between 10 and 60 times the normal potassium content of extracellular fluids. Such experiments were performed on different species and different excitable cells: by Curtis and Cole (1942) on giant nerve fibers of the squid; by Ling and Gerard (1950) and Adrian (1956) on striated frog muscle fibers; and by Huxley and the author (1951*b*) on single myelinated frog nerve fibers. The results showed clearly for all these excitable structures that the curve relating V_m with log $[K]_e$ approaches a straight line with a slope of 58-mV potential change for a tenfold increase of $[K]_e$ within the range of concentrations already mentioned. This behavior fits the Nernst equation for a potassium concentration cell, where $V_m = 58 \log ([K]_i/[K]_e)$ mV at 20° C., $[K]_i$ representing the potassium concentration of the cell plasma. The deviation of the experimental curve from the theoretical straight line and the flattening out of the curve at low $[K]_e$ was explained by permeabilities of the membrane to other ions, such as Cl^- and Na^+ which, although smaller than the potassium permeability, are not negligible and will prevent an increase of V_m to infinity at low $[K]_e$. Furthermore, Hodgkin and Katz (1949), basing on the constant field theory of Goldman (1943), obtained an equation fitting the experimental values quite well if the relative values for the permeability coefficients for K^+, Na^+, and Cl^- were chosen appropriately. Everything seemed consistent with the original view of Bernstein as far as the resting potential (RP) was concerned, and there was general agreement among physiologists that the RP is a PD of a potassium concentration cell, somewhat modified by a small but not negligible

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membrane permeability to other cations and anions; recent textbooks have adopted this explanation. Teachers as well as students like it for its simplicity. I reluctantly present new experiments showing that this easy explanation does not hold for Ranvier nodes and is possibly different for all excitable structures. The reader will forgive me, however, realizing that only strong experimental evidence has forced me to modify my own belief. This evidence and the reasons why other investigators were unable to obtain similar results are presented below.

METHODS

In previous experiments on the influence of altered $[K]_e$ on membrane potential, care was taken to wait for equilibration supposed to be complete if the newly acquired V_m remained constant for minutes. As all methods of investigation gave no possibility of changing the external concentration within a reproducible and very short time interval, the initial phase of adaptation was either not recorded or was considered irrelevant. The new equilibrium, therefore, was not only the result of a change of the PD of the potassium concentration cell, but shifts of anions, particularly of chloride, were likely to have occurred within the same time. Only Huxley and Stämpfli (1951*b*) made efforts to evaluate the rate of change of V_m and to compare it with the concentration change, calculated under the assumption of immediate mixing of the inflowing liquid with the volume of fluid contained in the trough where the nodes under investigation were immersed. These investigators came to the conclusion that the resting potential followed the calculated concentration change within less than 1 second if $[K]_e$ was increased from normal (2.5 mM/l.) to an eightfold value. Very few experiments of that type were performed, however. Their statement that "the effect of a change in ionic composition of the medium develops in a time of the order of one second" and that "the changes in resting potential are consistent with its being a diffusion potential across a membrane, permeable to K and to a smaller degree to Cl or Na ions" seems therefore only partly justified and is subject to correction after consideration of the results presented in this paper.

For the present investigations the "circulated-node" technique was used throughout (Stämpfli, 1956). This method consists in isolating a single Ranvier node from its neighbors by placing it in a stream of circulating fluid passing through a narrow polyethylene tube and drying the internodes on either side of it in air gaps (FIGURE 1). The other nodes of the dissected fiber are situated in troughs containing 0.2 per cent cocaine-Ringer's solution on both sides. They keep their membrane potential despite their inexcitability. Within the air gaps the external fluid dries until a film of about 1 μ remains. Because of the high longitudinal resistance of this film, any difference in potential developing between the "circulated" node in the middle and the neighboring nodes can therefore be recorded between the polyethylene tube and one of the neighboring troughs by means of silver-silver chloride electrodes connected to the respective liquids by saturated KCl-agar bridges. The recorded PD corresponds to the full membrane potential difference between the nodes multiplied with a short-circuiting factor. The higher the external longitudinal resistance in the air gap, the more this factor will approach unity. The approximate value of the short-circuiting factor can be determined by destroying the middle node at the end of an experiment by a strong positive pulse and by assuming a normal membrane potential of 68 to 71 mV (according to Huxley and Stämpfli, 1951*a*) for the measured potential drop recorded after destruction of the membrane. The short-circuiting factors determined by this method vary between 0.5 and 0.8. Determinations of this kind are possible, however, only if the resting level in normal Ringer's solution remains constant during the experiment and if the behavior of the membrane leaves no doubt as to the good condition of the nerve fiber.

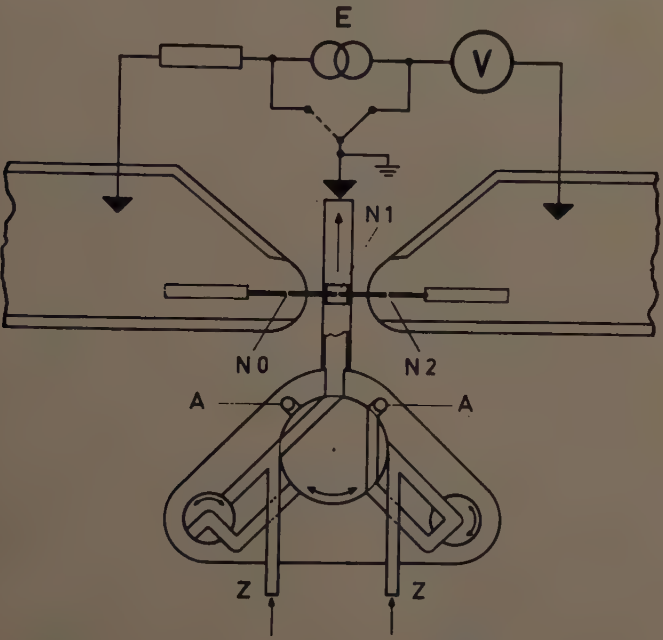

FIGURE 1. Method of the "circulated" node. A single nerve fiber of *Rana esculenta* crosses 2 air gaps between the circulating fluid of a polyethylene tube and 2 pools of 0.2 per cent cocaine-Ringer's solution containing the unexcitable nodes N_0 and N_2. The node under investigation (N_1) is placed in the axial stream of fluid circulating along the middle tube. Ag-AgCl-KCl agar electrodes are used to pass current across the nodal membrane of N_1 and to record its membrane potential changes by a high-impedance mV recording pen. N_2 serves as reference node. E = mV-calibrator, either as current source or calibrator for recording instrument V. Stopcock for rapid change of circulating fluid, with Z = inflow of solution into stopcock. A = outflow of liquid if unused borehole is washed with next test solution. Distance between stopcock and node is 3 mm.

Measurements were started after allowing the internodes to dry in the air gaps within 20 to 30 min. Changes of ionic concentration were obtained by switching from 1 circulating solution to another by means of a special stopcock situated only 3 mm. upstream from the node. The change of concentration was found to be complete within approximately 0.2 sec. Electrotonic currents could be imposed upon the nodal membrane by means of a stimulator or of a voltage calibrator across 1 air gap, and the ensuing change in membrane potential could be recorded with a high-impedance DC amplifier and a suitable recording pen or an oscilloscope across the other air gap. This arrangement also allowed the possibility of obtaining current-voltage curves of the membrane (Stämpfli, 1958*a*).

RESULTS

All experimental data and figures presented hereafter will be given as the inside potential of the nerve fiber, despite the use of external electrodes. Comparison with other data obtained with intracellular electrodes will thus be easier. On the other hand, the membrane potential will be spoken of as a positive quantity despite its negative sign. If it is said to increase, it becomes more negative, and the potential tracings will move downward to the negative side. Depolarizations, reductions of V_m, or potential drops, on the contrary, make the potential smaller and therefore less negative. Hence the recording pen will show an upward movement. FIGURE 2 gives a general orientation as to how the recordings must be interpreted and how current-voltage curves were obtained. One should remember that the full concentration change applied in any of the figures requires less than 1 sec. to complete, and that any further change in membrane potential must be due to adaptation processes that are independent of the time course of the change of $[K]_e$.

The Time Course of the Potential Change after a Sudden Increase of $[K]_e$

If $[K]_e$ is suddenly increased (within 1 sec. or less), the onset of depolarization varies considerably from one fiber to another and even for the same fiber if it is submitted to strong variations of potassium, sodium, or chloride concentrations during the experiment. If the dissection is carried out rapidly and with the utmost care, and if the node in the central polyethylene tube presents no sign of distention, small changes of $[K]_e$ up to 20 mM/l. or, in a few exceptional experiments, even up to 40 mM K/l., produce only small and slowly progressing depolarizations. At a critical concentration, however, a sudden depolarization is observed, undershooting the expected value of V_m for a potassium equilibrium and regressing to it within 5 to 30 sec.

Other fibers, however, having been dissected less successfully or taken from winter or spring frogs, react immediately with depolarizations to the theoretical value or less. For the moment, the methods used are not sufficiently accurate to enable me to state whether their rate of potential change corresponds exactly to the change in concentration, but if there is any difference it must be very small.

Badly dissected or distended fibers still differ from this behavior in presenting first a rapid depolarization and then a slow and long-lasting equilibration taking 1 min. or more. If the original small $[K]_e$ is again applied, repolarization takes the same time course, sometimes, however, without reaching the original level of V_m again. Such fibers usually tend to depolarize continuously. Their membrane potential, as measured by electric destruction of the nodal membrane, is low. The potassium concentration needed to depolarize the membrane to zero is smaller than 115 mM (isotonic KCl). We therefore suppose

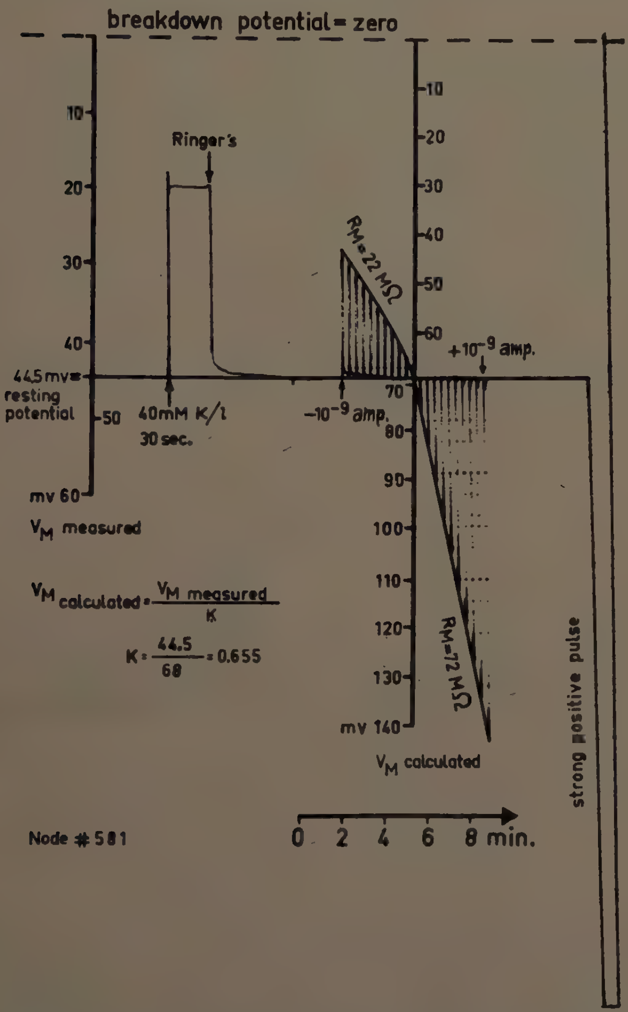

FIGURE 2. Example of recording membrane potential changes due to a sudden temporary increase of $[K]_e$ from 2.5 mM to 40 mM/l. and of a current-voltage curve. At the end of the experiment the membrane is destroyed by a strong positive pulse (see Stämpfli and Willi, 1957). The potential drops by 44.5 mV after destruction. The node is assumed to be normal and to have a V_m of 68 mV. The short-circuiting factor, having reduced the measured potential to 45 mV, can thus be calculated and the scale of V_m corrected accordingly. The current-voltage curve is recorded at reduced speed (compare 30 sec. of depolarization with time scale at the bottom of the figure). Stepwise decreasing cathodal pulses of 5-sec. duration are imposed to the membrane with current-free intervals of 15 sec. After reaching zero current intensity the same steps are applied in reversed direction up to a final value of 10^{-9} amp. Every step corresponds to approximately 10^{-10}-amp. membrane current (see under DISCUSSION). Note the steep depolarization and the kink due to rectification near resting potential typical for nodes of Condition 1. (Motor fiber of female *R. esculenta* at 21° C.)

that these nodes have lost a part of their intracellular potassium during dissection and mounting in the apparatus, probably under the influence of some damage to the node or to its myelin sheath. Results obtained on such fibers were therefore discarded.

We conclude from these preliminary observations that Ranvier nodes can exist in three different conditions as far as their reaction to a sudden increase of $[K]_e$ is concerned.

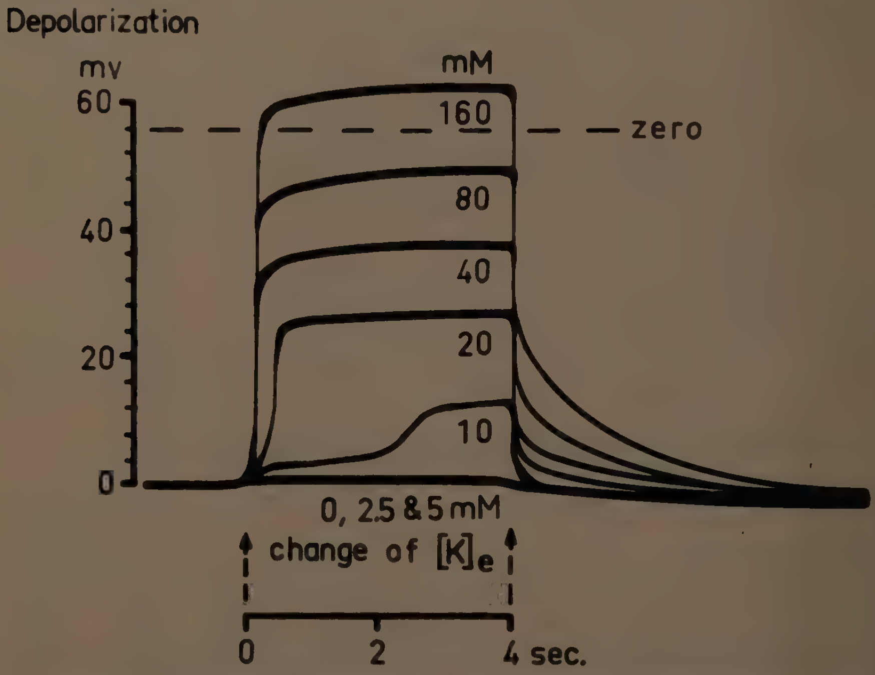

FIGURE 3. Time course of depolarizations caused by a sudden increase of external potassium concentration $[K]_e$ lasting for 4 sec.

Note slow depolarization at 10 and 20 mM K/l., despite the rapid change of $[K]_e$ completed within 0.2 sec. and the absence of any depolarization at 5 mM or of polarization at zero mM K. The node is barely sensitive to potassium if the membrane potential has not been reduced by a strong action of K. Oscillographic records superimposed. Ordinate: measured potential deflections, uncorrected for short-circuiting factor. Dotted line: potential after electric breakdown of node equals zero. (Male *R. esculenta* at 21° C.)

Condition 1. Relative lack of sensitivity to K^+ which is incompatible with the membrane theory of Bernstein, provided that a critical value of $[K]_e$ is not exceeded (FIGURE 3). This value usually lies between 5 and 20 mM $[K]_e$/l. It is difficult to state the percentage of nodes belonging to Condition 1 encountered in our experiments. The reason is that many of the nodes shift to Condition 2 if kept for a longer period in the apparatus or submitted repeatedly to raised $[K]_e$. Very striking examples of Condition 1 where even 40 mM $[K]_e$ can be withstood for one-half minute without a sudden shift to Condition 2 are very rare, but the steady improvement of our experimental technique, particularly of dissecting more rapidly without damaging the fibers and mounting the fibers in the apparatus without distention, has continuously increased the percentage of fibers showing a sudden change of $[K]_e$ sensitiveness around 10 mM $[K]_e$/l. We believe that obtaining Condition 1 fibers depends merely on careful and quick dissection, and that in fact all fibers *in vivo* are in Condition 1.

Condition 2. Nodes representing this class react immediately to increased $[K]_e$ in the way observed by Huxley and Stämpfli (1951*b*). However, they can behave exactly like nodes of Condition 1 if polarized by moderate anelectrotonus. Nodes of Condition 1, on the other hand, can be induced to behave like Condition 2 nodes under the influence of moderate catelectrotonus.

Condition 3. Nodes in Conditions 1 or 2 can deteriorate in long-lasting experiments with frequent application of unphysiological ion concentrations, such as high $[K]_e$ or $[Na]_e$ or low $[Ca]_e$. Deterioration can also occur after strong and long-lasting polarizations of the membrane to approximately twice the resting potential. The membrane of such nodes must have partly lost its selective permeability by formation of unspecific leaks. This conclusion is confirmed by current-voltage curves revealing small rectification and low membrane resistance.

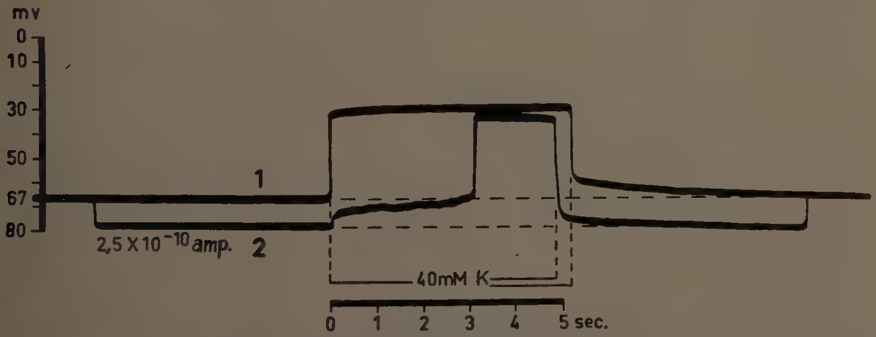

FIGURE 4. Oscillographic recording of depolarizations of a single Ranvier node due to an increase of $[K]_e$ from 2.5 to 40 mM under normal conditions (tracing 1) and an anelectrotonus of 2.5×10^{-10} amp., increasing V_m by 12 mV.

Note shift from Condition 1 in anelectrotonus to Condition 2 after a slow depolarization bringing V_m to the critical level of instability. From Stämpfli, 1958*a*; reproduced by permission from *Helvetica Physiologica et Pharmacologica Acta.*

Potassium Sensitiveness of Nodes as Function of V_m

The observations mentioned in the previous section indicate a V_m-dependent action of $[K]_e$-changes. FIGURE 4 shows a typical example of inhibited potassium sensitiveness of a node due to moderate polarization of the nodal membrane. The node gives an immediate depolarization representing Condition 2 at normal RP (tracing 1). An increase of V_m by 12 mV, however, hinders the membrane from responding in the same way during 3 sec., the time needed to depolarize the membrane slowly to a critical level where Condition 2 is again operating (tracing 2). There is a slow and irregular rise to the critical level. The irregularities are due to "noise" of the membrane; that is, there is a marked tendency to random small variations of V_m. This behavior is typical for a maintained high V_m despite of an increased $[K]_e$. It is also observed in Condition 1 fibers if they are sufficiently insensitive to $[K]_e$ and if the potassium concentration tested is not far from the critical concentration, converting the membrane to Condition 2.

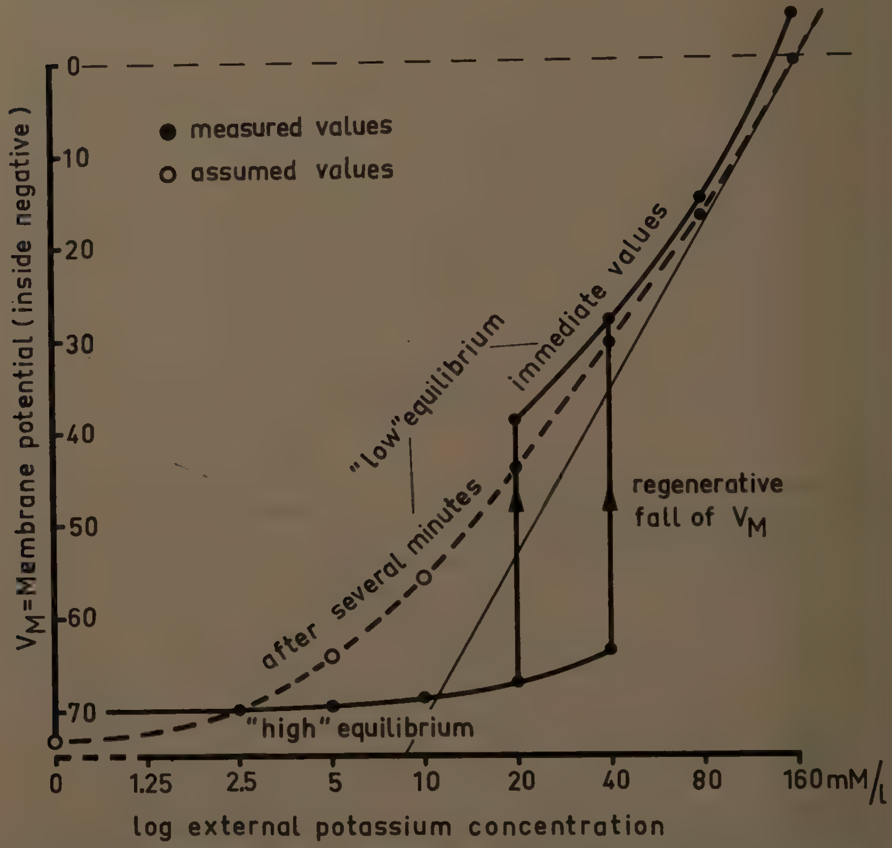

FIGURE 5. Membrane potential versus log $[K]_e$ of a single Ranvier node. "Immediate" values were measured about 2 sec. after the beginning of the concentration change or after regenerative fall to the "low" equilibrium. The dotted curve represents the values obtained after long equilibration of several minutes. It was necessary to assume some points of this curve because of the extreme stability of the "high" equilibrium, which caused spontaneous repolarization at 5 and 10 mM $[K]_e$ even when cathodal shocks had displaced V_m to the "low" value. (Motor fiber of female *R. esculenta* at 21° C.)

The curves of V_m versus log $[K]_e$ look different for Condition 1 and Condition 2 fibers. The experiment shown in FIGURE 5 was conducted with an exceptional Condition 1 node. The "immediate values" of depolarization correspond to the change in potential observed either 2 sec. after switching to an increased $[K]_e$ or 2 sec. after having reached the low level, if a delayed depolarization occurred. V_m remained at the "high" equilibrium in 20 mM $[K]_e$/l. The "low" value for this $[K]_e$ was obtained by applying a short cathodal pulse to the node causing an immediate shift to the peak of depolarization and a slow repolarization within 15 sec. This intermediate potential corresponds to the equilibrium observed by Huxley and Stämpfli (1951*b*). At 40 mM $[K]_e$ V_m decreased slowly for about 10 sec. and then fell spontaneously to the "low" equilibrium. The discontinuity of the curve connecting the values measured after 2 sec. convincingly shows 2 membrane conditions. The smooth dotted curve, however, obtained after several minutes of raised $[K]_e$, corresponds to the one presented by Huxley and Stämpfli (1951*b*), approaching the theoretical 58 mV line in continuous fashion. At 5 and 10 mM $[K]_e$ it was impossible to obtain a regenerative fall of V_m to the low level by cathodal shocks, but they produced unusually strong deflections of V_m toward the low level that could be explained only by shifts of the membrane equilibrium. The stability of the "high" equilibrium was thus strong enough to shift V_m spontaneously back to the high level after being pushed down to the low one by a short catelectrotonus.

The Current-Voltage Relation of Ranvier Nodes in Ringer's Solution

If a current-voltage curve of a node is recorded in normal $[K]_e$ of Ringer's solution according to the technique explained in FIGURE 2, the three conditions of nodes mentioned above can be distinguished again. Condition 1 nodes, for example the one used in FIGURE 5, show a bending of the curve, caused by a change in membrane resistance or rectification only under influence of catelectrotonus, that is, below RP (FIGURE 6). Condition 2 curves, however, bend near or at the resting potential (see FIGURE 2). Condition 3 nodes at last show only moderate rectification in anelectrotonus, that is, above RP. The curves are steep compared with normal nodes, revealing a small membrane resistance.

The Current-Voltage Relation at High $[K]_e$

Attempts were made to measure current-voltage curves at increased $[K]_e$; a typical result is shown in FIGURE 7. It is characteristic for nodes of Conditions 1 and 2 as the difference between them vanishes after prolonged treatment with high $[K]_e$. At negative (outward) currents small linear deflections of membrane potential are obtained. This linear response is also observed for smaller positive (inward) currents, but at higher intensities the changes of V_m become increasingly nonlinear. Eventually, at a critical level, the potential recorder slows down at a first low value and then continues to move with increasing velocity from there to a new stable value at a much higher V_m, situated above the original resting potential of the fiber. If the polarizing current is now switched off, V_m falls back, undershooting the former low level and returning to it within 15 or 20 sec. Such peaks of depolarization are always visible if V_m suddenly drops from Condition 1 to Condition 2, not only if the polarizing current, having shifted V_m to Condition 1, is opened but also when a fiber at Condition 1 is suddenly treated with a $[K]_e$ sufficiently strong to produce a regenerative fall of V_m to a potassium potential representing Condition 2 (see FIGURE 5).

The shape of the current-voltage curve at high $[K]_e$ of FIGURE 7 may be compared with the one at normal $[K]_e$ (FIGURE 6). Its linear parts differ from each other in much the same way, revealing a lower membrane resistance at catelectrotonus and a higher one at anelectrotonus. The essential difference, however, is the sudden shift that takes place within the nonlinear part of the curve. This shift is definitely caused by a sudden new equilibrium condition of the membrane. Therefore, the amplitude of the membrane potential deflection cannot be proportional to the membrane resistance R_M alone, since it includes a potential shift due to formation of a new ionic equilibrium. This characteristic of the current-voltage curve will be discussed later.

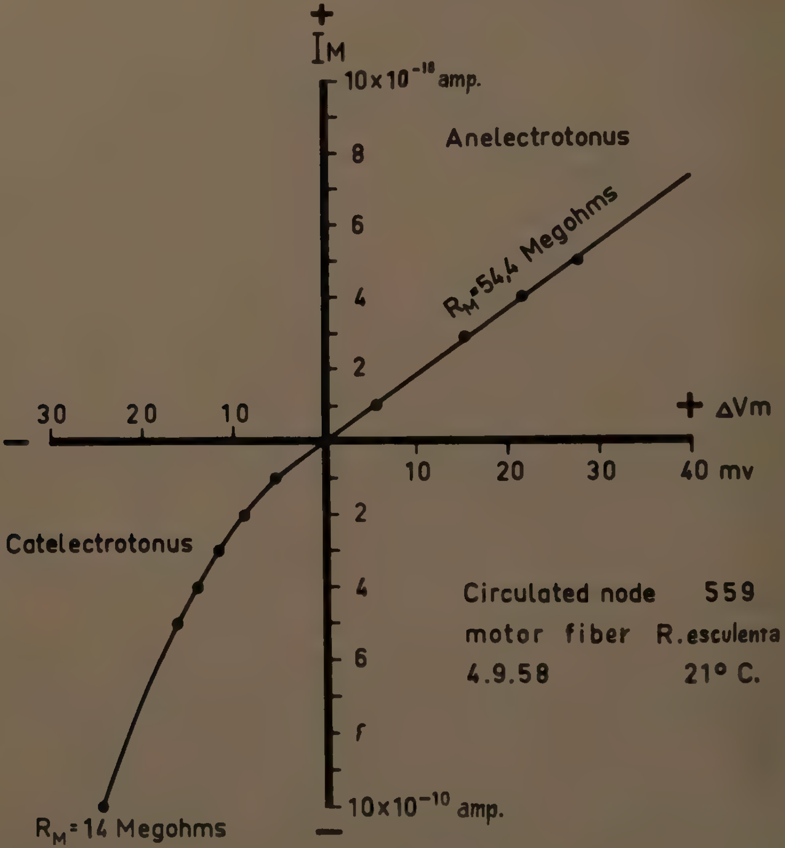

FIGURE 6. Current-voltage relation of a Condition-1 node. The measured points were obtained by electrotonus of 5-sec. duration, separated by long intervals. The procedure of recording the curve was avoided to protect the node from excessive current flow. Same experiment as in FIGURE 4. See under DISCUSSION as regards the calculated membrane resistances.

Similar curves showing the typical shift of equilibria can be obtained by a different procedure, shown in FIGURE 8. Electrotonic currents are applied at normal $[K]_e$ in steps of 2.5×10^{-10} amp. and with intervals of 1 min.; each current pulse lasts 30 sec. Between the tenth and the twentieth seconds of the pulse, $[K]_e$ is suddenly raised to 40 mM/l., and the change of V_m is recorded. The depolarizations obtained by this method again reach a low level in catelectrotonus and fail to do so as soon as the membrane potential has reached a critical value of anelectrotonic polarization. The node used for this experiment was in Condition 2. The location of the nonlinear portion of the current-voltage curve at RP level favors this view.

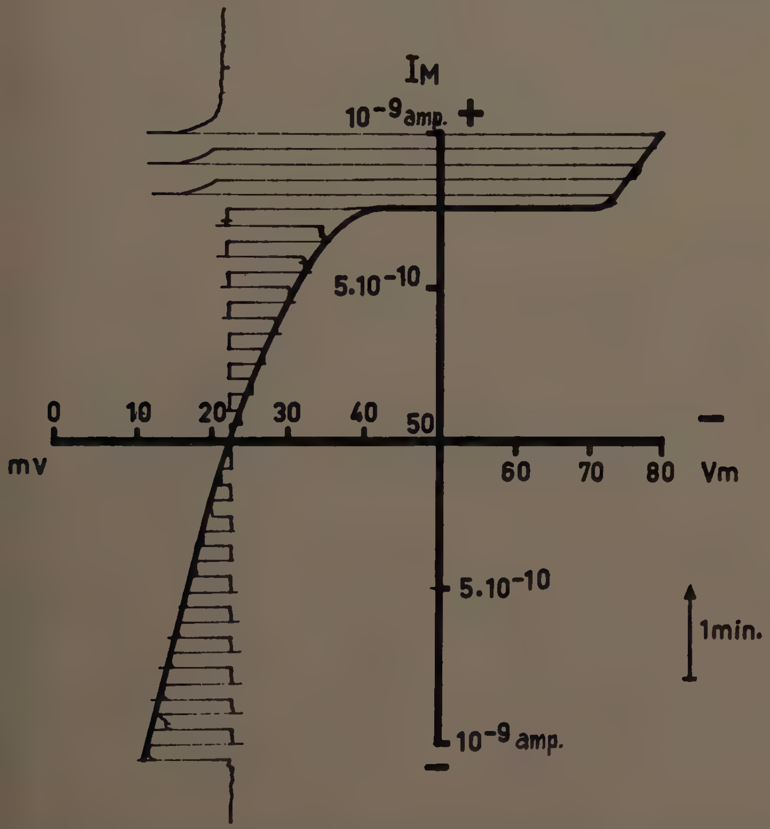

Figure 7. Current-voltage relation measured at $[K]_e$ = 40 mM/l. Horizontal axis: membrane potential V_m not corrected for short-circuiting factor $k = 50/68 = 0.74$. The curve starts at equilibrium potential of the membrane at $[K]_e$ = 40 mM, 28 mV below resting potential. Vertical axis: membrane current calculated under the assumption of a total circuit resistance of 200 megohms (see under Discussion). For the recording technique see caption to figure 2. Note thickening of upstroke, indicating slow movement of the recording pen at $+8 \times 10^{-10}$ amp. and subsequent new rapid increase of V_m, indicating a shift to the "high" membrane equilibrium. (Female *R. esculenta* at 20° C.)

Similar experiments were carried out with other $[K]_e$ showing the same behavior within a range of 10 to 160 mM K/l. The critical potential where the shift between the two equilibria occurs is the same, regardless of the $[K]_e$ employed (Stämpfli, 1958*a*). The anelectrotonic currents necessary for reaching this level must be increased at high $[K]_e$. This is due partly to the greater potential difference existing between the level of depolarization and the critical potential, and partly to the reduction of R_M demanding stronger currents for displacing V_m to the critical value. It is interesting to notice that, as soon as the "high" equilibrium is obtained by anelectrotonus, the new equilibrium will be maintained at lower intensities of polarizing currents. Therefore, the current-voltage curve looks different if polarizing currents are not switched off after reaching the high level, but are reduced in a continuous manner. V_m will then gradually fall on the "high" equilibrium curve until the critical level is approached and a sudden drop to the old level is observed. This is another confirmation of the view that two stable states of the membrane are possible at high $[K]_e$ and that either one can exist only within a limited range of V_m. If by electrotonus V_m is pushed to the critical limit, where one equilibrium becomes unstable, a sudden self-sustained shift to the other equilibrium occurs. This applies in both directions from the "low" to the "high" equilibrium, and vice versa.

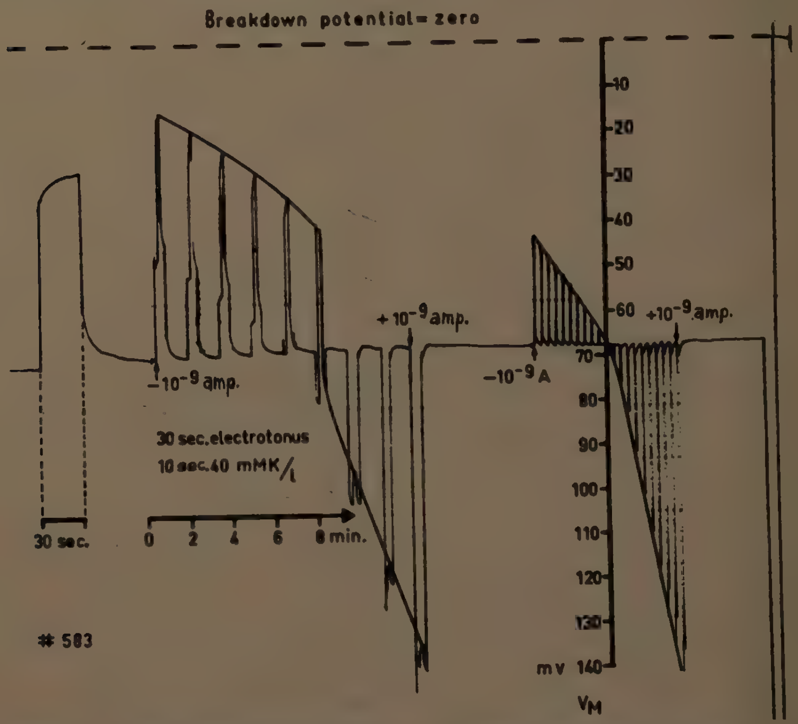

FIGURE 8. Depolarization by 40 mM $[K]_e$/l. at different membrane potentials. Current pulses of 30-sec. duration are given in steps of 2.5 × 10^{-10} amp. separated by an interval of 1 min. From the tenth to twentieth second of each pulse 40 mM K-Ringer's is applied. The curve connecting the maxima of depolarization resembles the current-voltage curve of FIGURE 6. Thus there is no essential difference between curves obtained at complete equilibration with $[K]_e$ by electrotonus and others where the electrotonus is applied at normal $[K]_e$ and short concentration pulses are given. (Female *R. esculenta* at 21° C.)

The "Low" Equilibrium

The results thus far indicate that a "low" equilibrium exists near the theoretical potassium potential. The curve presented in FIGURE 5, which was obtained after long equilibration with high $[K]_e$, resembles the one of Huxley and Stämpfli (1951*b*), and is taken as evidence that the nodal membrane behaves like a K electrode; hence the "low" equilibrium can be accepted as a potassium potential. At this equilibrium the change of V_m with varying $[K]_e$ meets the requirements of the membrane-potential theory of Bernstein (1902), with its recent modifications mentioned above. At this low-equilibrium state the membrane resistance depends on $[K]_e$. At the high equilibrium, however, the influence of $[K]_e$ is strongly reduced in every respect, and changes of $[K]_e$ produce only minor variations of V_m and R_M.

The Nature of the "High" Equilibrium

A possible answer to the question, which potential-producing mechanism might operate at the high equilibrium, was given by Hodgkin (see Stämpfli, 1958*a*). He compared the nodal membrane to the one of the squid giant nerve fiber, studied extensively by himself and with Huxley and Katz (1949). This membrane changes its conductivity to potassium ions g_K inversely with the membrane potential. Hodgkin supposed, from some of the results presented in this paper, that obtaining 2 membrane equilibria in nodes was due to a similar dependence of g_K. In the squid, g_K is low at high V_m. Why not assume that, in myelinated fibers, g_K at resting potential is so low that other ionic conductances take over in determining V_m? The conductance for chloride g_{Cl} in squid fibers was found to remain constant over a wide range of V_m. If this holds for myelinated nerve as well, and if g_{Cl} at resting potential is considerably higher than g_K, the resting potential will be a chloride potential. This explains the relative insensitiviness to changes of $[K]_e$, which will become effective only if they succeed in lowering V_m to a point where g_K overpowers g_{Cl} and determines the membrane potential. If this point is reached, any decrease of V_m will increase g_K and therefore further reduce V_m. This sudden fall of V_m toward the theoretical potassium equilibrium resembles the rising phase of the action potential, where the regenerative linkage between g_{Na} and V_m leads to an accelerated depolarization toward the sodium equilibrium potential.

On the other hand, polarization of the potassium-sensitive membrane will reduce g_K and the influence of potassium on membrane potential. At a critical level g_{Cl} will take over and move the membrane potential toward the chloride equilibrium, which is well above the one for potassium, as the chloride concentration of the external medium remains unchanged in our experiments and as only K^+ is substituted for Na. This idea was corroborated by a simple mathematical analysis worked out by A. L. Hodgkin (see Stämpfli, 1958*a*), who showed that under the assumptions mentioned above and a few additional ones, three membrane equilibria are possible at high $[K]_e$: a stable one near the theoretical potassium potential, an unstable one between the potassium and chloride potential, and a third stable one near the chloride potential. This explanation of our results seems convincing and simple. Its validity, however, depends on experimental evidence proving that the "high" equilibrium is governed by chloride ions.

The method of proving the influence of a particular ion on V_m has been shown for K in the preceding sections. We tried to use it for chloride as well. Increases of chloride ions in the external fluid can be obtained only by making it

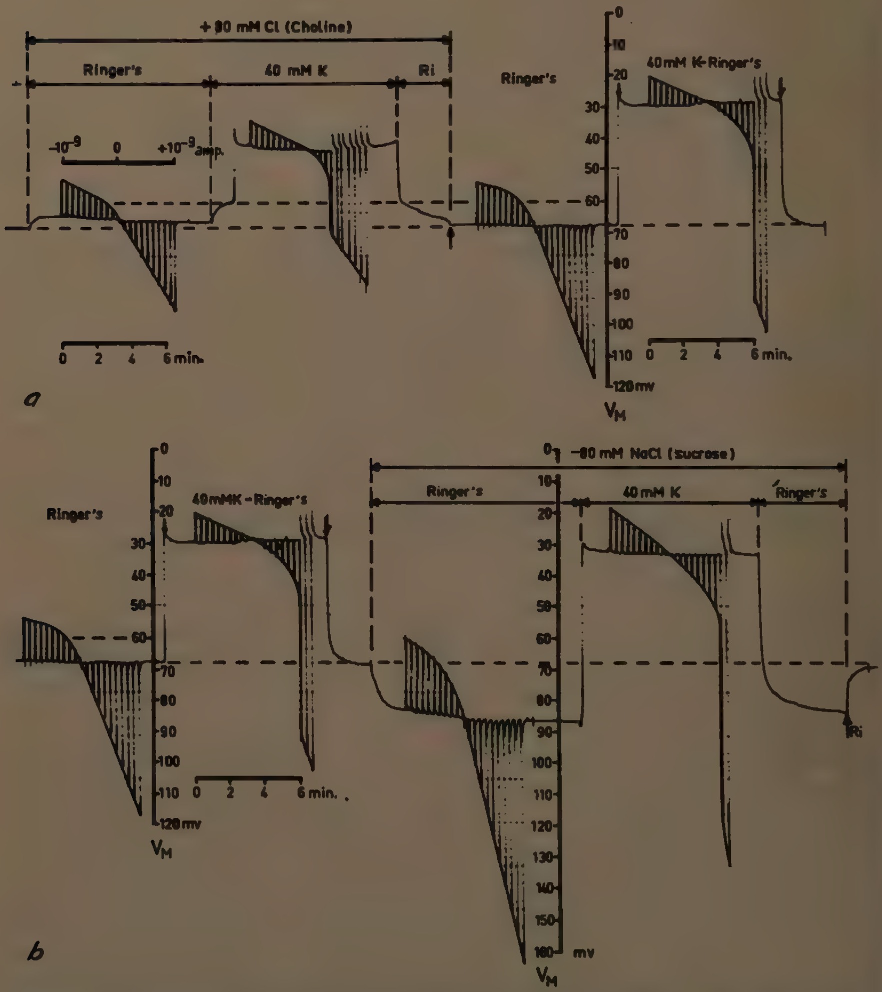

FIGURE 9. Stabilizing action of chloride: original membrane potential recordings of current-voltage curves: (*a*) at increased $[Cl]_e$ in Ringer's and 40 mM $[K]_e$, and (*b*) at lowered $[Cl]_e$ in Ringer's and 40 mM $[K]_e$. For comparison with normal Ringer's and 40 mM $[K]_e$, the respective current-voltage curves are reproduced in both parts of the figure; parts *a* and *b* thus overlap. Note depolarization by an increase of $[Cl]_e$, reduced R_M in anelectrotonus, and shift of V_m to the "high" equilibrium at low polarization currents. The contrary is seen at reduced $[Cl]_e$. Only at high $[Cl]_e$ the slow depolarization due to increased stability of the "high" equilibrium and the critical level of instability (dotted line) can be recognized easily. Ordinate-membrane potential in mV, corrected for short-circuiting factor. (Female *R. esculenta* at 21° C.)

hypertonic and using an anion of unspecific action. Choline, which is generally accepted as unspecific, since it is too large to penetrate the membrane, was used. Adding 80 mM choline chloride to Ringer's solution should increase V_m, if the RP is a chloride potential. However, the addition of chloride was invariably found to depolarize the membrane. Accordingly, lowering the external sodium chloride concentration by the same amount and substituting sucrose had a strong polarizing action (+19 mV!) that was far stronger than the one obtained by removal of the same amount of Na alone (+4 mV). Hodgkin's explanation seems difficult to accept on account of these results, although a strong action of Cl on V_m cannot be denied. Therefore, experiments using the same variations of $[Cl]_e$ were extended to current-voltage curve determinations (FIGURE 9*a* and *b*).

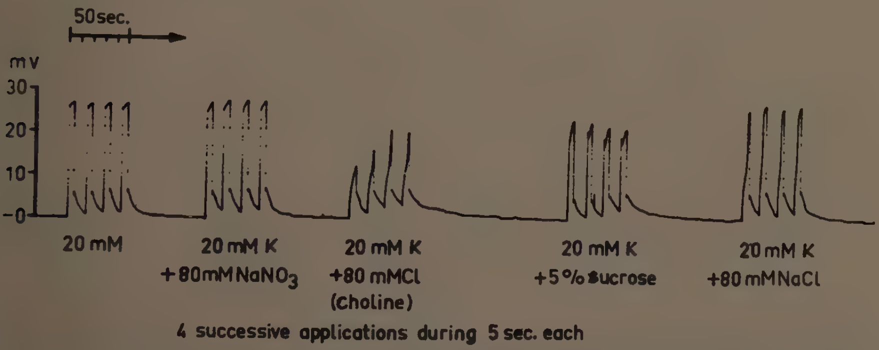

FIGURE 10. Stabilizing action of Cl ions: recording of 4 successive depolarizations due to $[K]_e = 20$ mM/l. with and without addition of anions or equivalent amounts of sucrose. Only added chloride slows the initial phase of depolarization. Its action is strongest at the first depolarization. Repeated depolarizations thus reduce the stability of the "high" equilibrium and favor the "low" one. NaCl is less active than choline chloride, as addition of Na tends to lower V_m. Nevertheless, the depolarization consists of 2 distinct rapid phases with a slow intermediate phase, due to a third, unstable equilibrium (see text). Ordinate = depolarization in mV, corrected for short-circuiting factor. (Female *R. esculenta* at 22° C.)

Addition of 80 mM Cl/l. by adding an adequate amount of choline chloride favors the shift from the "low" to the "high" equilibrium (FIGURE 9*a*). This "stabilizing action" of increased $[Cl]_e$ is also revealed by the time course of depolarization, which is characterized by a slow beginning and a sudden acceleration under the influence of the regenerating increase of g_K. The time course of depolarization at normal $[Cl]_e$ barely shows a first slow phase. The opposite phenomenon, namely, increased stability of the "low" equilibrium and delayed shift to the "high" equilibrium in anelectrotonus, is seen after removal of 80 mM NaCl and substitution of sucrose (FIGURE 9*b*). This effect is not due to removing Na. Normal curves are obtained if Na is substituted by choline.

These two experiments favor the view that the "high" equilibrium has something to do with Cl ions despite the unexpected results at resting potential level.

Another proof for the "stabilizing action" of Cl ions is given in FIGURE 10. The experiment represented on it was undertaken during another investigation, dealing with aftereffects of depolarization and their possible summation. Four successive changes of $[K]_e$ to 20 mM/l. of 5-sec. duration were made at 10-sec. intervals. This procedure was repeated with addition of 80 mM/l. $NaNO_3$, choline chloride, NaCl, or 5 per cent sucrose (making up for the increase of osmotic pressure). The time course of depolarization clearly shows a slow phase of depolarization if 80 mM choline chloride or NaCl are added. $NaNO_3$ has no effect, sucrose possibly a small one.

The reduced stability of the "low" (potassium) equilibrium at increased $[Cl]_e$ is finally seen in FIGURE 11. After depolarization by 20 mM K/l., polarizing current pulses were applied. An intensity of 2.5×10^{-10} amp. failed to shift the "low" equilibrium to the "high" one. If, however, 80 mM of choline chloride or NaCl was added, pulses of 1.5×10^{-10} amp. were sufficient to produce a shift.

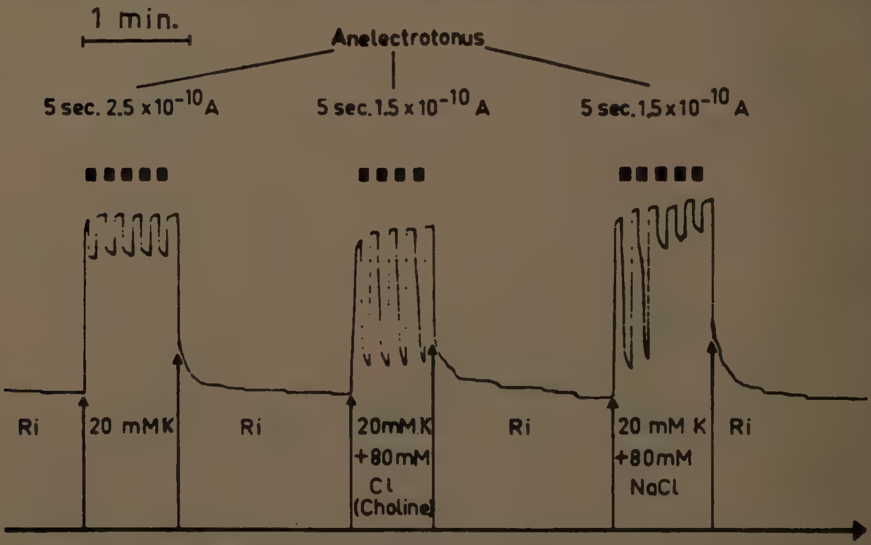

FIGURE 11. Stabilizing action of Cl ions: polarizing pulses of 2.5×10^{-10} are unable to shift V_m from "low" to "high" equilibrium. After addition of Cl, however, weaker pulses of 1.5×10^{-10} are able to do it. Note again the loss of stability of the "high" level at prolonged action of depolarization. Same experiment and coordinates as in FIGURE 10.

DISCUSSION

Some critical remarks concerning the method should be made before discussing the results. Could they be explained by methodical errors? The present method differs from that used by Huxley and Stämpfli (1951*b*) in several respects, particularly by using air gaps. The drying of internodes is unphysiological and could alter the properties of the nodes situated on either side of the gap. Tasaki (1953) also used air gaps in many of his experiments. He finds that drying the internode does not affect the neighboring nodes. Our own results have always confirmed this view. The shape and size of action potentials and action currents are exactly the same, independent of the use of air gaps, oil gaps, or partitions with small capillaries if corrections are made for the short-circuiting factor. For technical reasons we have not yet tried to confirm our present results with a different arrangement, using two oil gaps or two capillary partitions instead of air gaps. We have never been able to prove an influence of the length of the air gap on the two equilibria. Variation of the short-circuiting factor influences electrotonically induced deflections of the membrane potential by the same proportion as the amplitude of depolarization for a given $[K]_e$. Only long-lasting experiments with a wide air gap show a reduction of the short-circuiting factor together with a very high resistance of the preparation after a certain time. This is due to desiccation of the fiber and to shrinking and increase of the axoplasm resistance within the air gap. Such preparations, however, can well be distinguished from normal ones and are always discarded.

Another question is whether the part of the internodes situated next to the "circulated" node within the polyethylene tube plays an important role. Some checks of the method with an internode in the tube instead of a node gave only insignificant changes of the transmyeline potential after raising $[K]_e$ and no sign of two equilibria in the straight current-voltage curves. The slope of the current-voltage curves was, however, almost the same as in the anodal part of a curve obtained with a node. The nodal region is only ½ μ wide, and it is likely that the internodal region determines the resistance if the nodal resistance, which is parallel, has grown to high values. The interpretation of the upper part of the current-voltage relation therefore seems doubtful. We suppose that the shape of the current-voltage curve may be distorted to a certain extent by the parallel resistance of the adjacent parts of the myelin sheath lying in the fluid of the central tube and forming a pathway for electrotonic currents. This distortion may cause an exaggerated straightening of the current-voltage curve in anodal regions and hinder the curve from flattening out still further under the influence of a possible further increase of membrane resistance due to anelectrotonus. Experiments are planned where the internodal region within the central tube can still be reduced. The current-voltage curves under anelectrotonus might then become less steep, revealing more efficient rectification. Under catelectrotonus the nodal resistance seems to drop strongly enough to impose its value despite the parallel myeline resistance. Bending of the current-voltage curve may therefore only correspond to the point at which the nodal resistance drops to values of the order of the parallel myelin resistance. However, the distortion, if existing at all, is probably of minor importance since the nonlinear part of the curve coincides with the potential level where the critical instability of the membrane equilibria occurs.

Polarization of electrodes cannot have caused our results. Experiments with two small glass capillaries instead of a nerve fiber give perfectly linear current-voltage curves with very little distortion of the applied rectangular currents. Changes of $[K]_e$ under these conditions cause small potential changes of the same order as observed when an internode is placed in the central tube. The magnitude of these potential changes is within the values for junction potentials published by Huxley and Stämpfli (1951*b*).

As mentioned before, the recorded potential differences between the "circulated" and the reference node in cocaine were reduced by the resistance of the external fluid film at the right side of the air gap. A similar shunt exists at the left, conducting a part of the applied electrotonic current. We have not tried to correct the currents for this, as the absolute value of current indicated on current-voltage relations was not measured but assumed from a few preliminary resistance measurements, showing that the total resistance of the current-carrying circuit is regularly near 200 megohms, including the 100-megohm series resistor. This result was confirmed by a few current measurements giving a current of approximately 10^{-9} amp. during the application of an emf of 200 mV. There must be an error of at least ±20 per cent in our absolute values of membrane current due to variations of the total resistance of the circuit with fiber diameter and the width of the air gaps. Correction for the shunting effect of the external fluid would therefore not have improved the accuracy of the method, all the more so as an important objection to the interpretation of current-voltage curves must still be made. These curves can give, in fact, only quantitative values for membrane resistance if the equilibrium potential of the membrane is not changed by the application of current or by the ensuing potential changes. Only in this case the change ΔV_m divided by the current corresponds to the membrane resistance, but our results prove the existence of three equilibria and the possibility of shifting from one equilibrium to another by means of electrotonus. This is the reason why accurate measurements of membrane currents seemed difficult to perform without eliminating one of the variables, for example, by clamping the membrane potential. On the other hand, the results obtained by measuring current-voltage curves were reproducible to such an extent as to be taken as qualitative evidence of two completely different stable states of the membrane.

All results presented in this paper favor the view that Ranvier nodes do not behave like potassium electrodes as long as they are in excellent condition and as long as the external potassium concentration is not increased to unphysiological values of ten or more times normal. This view is based on the following five experimental facts.

(1) Two distinctly different kinds of depolarization are observed after a sudden increase of $[K]_e$, namely (*a*), a small depolarization that tends to increase slowly to an equilibrium value situated near the original resting potential, and (*b*) a rapid depolarization of great amplitude corresponding to the one described by Huxley and Stämpfli (1951*b*), reaching an equilibrium at or near the theoretical value of a potassium electrode.

(2) The first of these states is stable only above a critical V_m. It changes into the second state (*b*) as soon as V_m has fallen to a critical level either spontaneously or under the influence of a cathodal impulse.

(3) The stability of state *a* depends on the presence of Ca ions (Stämpfli, 1958*b*) and of Cl ions. An excess of either one or both stabilizes the membrane at state *a* and reduces the stability of state *b*. Hence, state *b* can shift spontaneously to state *a* if Ca or Cl ions are added to the external medium.

(4) The time course and amplitude of depolarization caused by a given increase of $[K]_e$ vary with V_m. Under anelectrotonus, depolarization is slow and the amplitude small if the depolarization does not exceed the critical level situated near the maximum of nonlinearity of the current-voltage curve. At this level, a sudden depolarization of high amplitude results, overshooting temporarily the equilibrium reached later. Catelectrotonus applied previously

to raising $[K]_e$ will speed up and enhance depolarizations of type *a* but will have little influence on depolarizations of type *b*. Only the depolarizations observed under the influence of a slight reduction of V_m, either by impaired conditions of the nerve fiber or moderate catelectrotonus, comply with the behavior of a potassium-concentration cell in the presence of less powerful Na and Cl cells. At resting potential and above, some other potential-producing mechanism than the gradient of potassium concentrations must be operating.

(5) The membrane resistance estimated from current-voltage curves must be considerably higher at state *a* than at state *b*. $[K]_e$ influences the membrane at state *b*, but not or very little at state *a*.

Most of these experimental facts can be explained in terms of Hodgkin's conception of the interaction of a voltage-dependent potassium conductance g_K and a constant chloride conductance g_{Cl} exceeding g_K at resting potential level. Under this assumption and the additional one of practically the same equilibrium potential for Cl and K at normal $[K]_e$, mathematical treatment predicts three values of V_m satisfying equilibrium conditions (zero membrane current) if $[K]_e$ is increased, leaving $[Cl]_e$ unchanged (for further details see Stämpfli, 1958*b*). Two of these equilibria are stable: one near the equilibrium potential for Cl, the other near the equilibrium potential for K at the increased $[K]_e$. The third equilibrium is unstable and is situated between the two stable ones. The "low" equilibrium of our results would thus be governed by potassium ions (which is confirmed by our results), and the high one by chloride ions. Considerable difficulties, however, arise in proving this latter conclusion experimentally. Changes of $[Cl]_e$ affect the membrane potential and the membrane resistance in the way shown in FIGURE 8*a* and *b*. A sudden increase of $[Cl]_e$ lowers V_m, which contradicts the conception of a simple concentration cell. An excess of chloride further lowers R_M in the anelectrotonic range of the current-voltage curve, but increases it under catelectrotonus. Movements of chloride across the membrane are supposed to be outward under anelectrotonus and inward under catelectrotonus. Some difficulties are thus encountered in explaining how raising $[Cl]_e$, and therefore the chances for Cl to move inward, can increase the membrane resistance instead of lowering it. Hence the action of Cl on membrane potential and membrane resistance is paradoxical.

On the other hand, Cl acts in favor of the "high" equilibrium and counteracts the "low," as should be the case according to Hodgkin's hypothesis. At the present state of our experimental investigation on the action of Cl ions, it seems inadvisable to discuss at length the possible reasons for the strange actions of Cl on single nodes of Ranvier and to approve or to condemn the concept that their resting potential is a chloride potential. The considerable influence of Cl on characteristic membrane properties at resting level certainly deserves further attention. Even if the postulate of a chloride potential as basis of the normal resting potential of myelinated nerve fibers cannot be proved for the moment, our experimental results leave little room for doubt that chloride plays an important role.

We can finally answer the question: Is the resting potential of Ranvier nodes a potassium potential? Our experimental results clearly indicate that the answer must be a negative one.

SUMMARY

(1) Ranvier nodes of isolated frog nerve fibers show two stable states of membrane potential at increased $[K]_e$, a "high" one that is probably determined by Cl ions (Condition 1) and a "low" one near the theoretical potassium potential (Condition 2), as revealed by current-voltage experiments.

(2) These two conditions can be observed only by an improved technique: very careful dissection of the fibers and rapid change of external ion concentrations are most essential.

(3) Two stable states and an unstable one can be explained theoretically according to a proposition made by Hodgkin only if certain assumptions on the relative values of g_K and g_{Cl} and their dependence on membrane potential are fulfilled.

(4) However, some effects of Cl ions, for example depolarization by excess chloride, cannot be explained at present.

(5) The results show clearly that the RP of myelinated nerve fibers of frogs is not a potassium potential. They thus contradict Bernstein's membrane theory.

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ION MOVEMENTS STUDIED WITH SINGLE ISOLATED ELECTROPLAX*

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INTRODUCTION

The electric organs of certain fish are the most powerful bioelectric generators created by nature. The discharge of the electric organ of *Electrophorus electricus* L., the "electric eel," has a potential of 600 volts and an intensity of 1 amp. The electric potential generated by the cellular unit, the electroplax, is of the order of 0.14 V. Voltage, time relationships, and other electric characteristics of the unit are quite similar to those observed in action potentials of nerve and muscle. It is only their structural arrangement in series and in parallel that causes such a powerful discharge. The basic similarity between bioelectric potentials of electric tissue and those in nerve and muscle has been early recognized, and the electric characteristics have been studied for more than a century.

It has long been assumed that ion movements are the carriers of electric currents generated in living cells. Conducting tissues such as nerve and muscle, like other cells, maintain an unequal distribution of ions between their interior and the surrounding medium. Na^+ is highly concentrated in the outside fluid, K^+ in the cell interior. During an action potential the permeability changes, and the two ion species move according to their concentration gradients.

A question fundamental for the understanding of the generation of bioelectric currents is how a cell is able to change its permeability. A large body of evidence has accumulated indicating that the intervention of a chemical reaction is responsible for the change of permeability observed. For more than twenty years Nachmansohn has studied the biochemical aspects of the generation of bioelectric potentials using, since 1937, electric tissue for his investigations. This material is a particularly useful tool for chemical studies. In view of the power of the discharge and the highly specialized function of the tissue, the chemical events are in the range of measurement, whereas in ordinary nerve fibers chemical reactions associated with activity frequently are too small for detection. In his studies Nachmansohn established the sequence of energy transformations associated with bioelectric potentials; he correlated chemical and physical events in many ways and integrated acetylcholine into the metabolic pathways of conducting tissue. On the basis of these and other data, he attributes a primordial role to acetylcholine in the permeability change.[1]

The chemical nature of the permeability change during electric activity has recently found strong additional support by physical measurements. Hill

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and his associates found a strong positive heat coinciding with the action potential followed by a strong negative heat.[2] A high temperature coefficient of the action potential was found with the use of a single electroplax, the Q_{10} being 3.6; the energy of activation was found to be high (21,000 cal./mole).[3]

During the last few years data have been accumulated in Nachmansohn's laboratory in New York, N. Y., and in Chagas' laboratory in Rio de Janeiro, Brazil, in which isolated pieces of electric tissue containing two to three rows of electroplax were used. These studies were concerned with electric characteristics and, particularly, with the effects of compounds that are known to interfere with the metabolism of acetylcholine. Considerable difficulties, however, were encountered in the interpretation of the results due to the complexity of the preparation used. It is obviously impossible, in a multicellular preparation containing several types of membrane, to distinguish between the different types of membranes affected. It therefore appeared desirable to

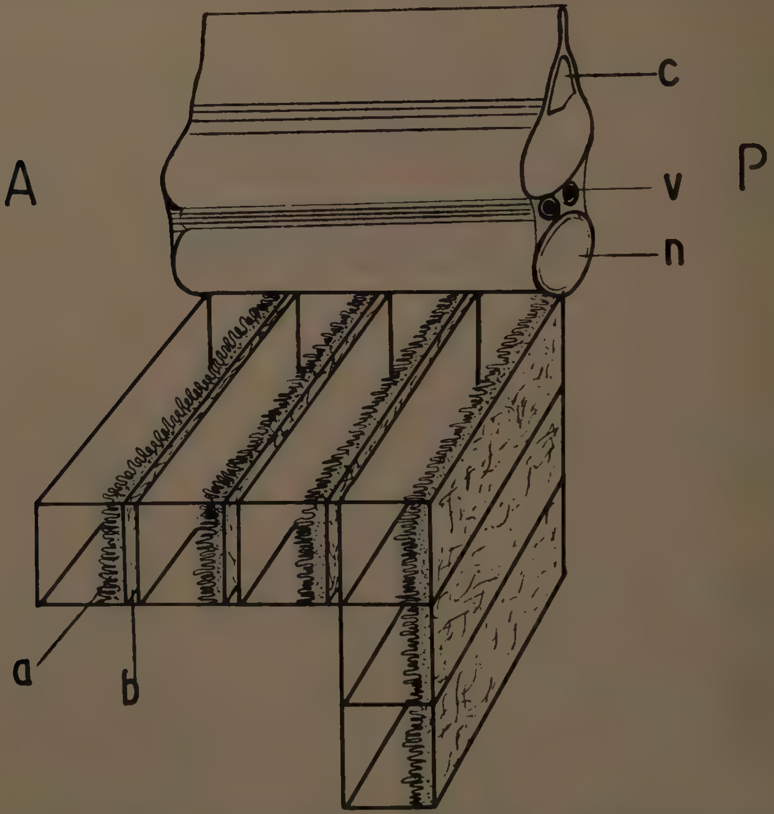

FIGURE 1. Schematic presentation of a fragment of electric organ (bundle of Sachs) of *Electrophorus electricus* L. The drawing shows the relationship between electroplax and compartments and the position of the conducting (caudal face: *b*) and nonconducting (rostral face: *a*) membranes. Although the drawing is not to scale, it shows that the ratio of extracellular over intracellular space is very high. Key: *A*, anterior (rostral) end; *P*, posterior (caudal) end; *n*, swim bladder; *v*, dorsal blood vessel; *c*, spinal cord.

devise a method in which a single cellular unit could be used for studying the properties of the cellular membrane. The electroplax of the eel is particularly favorable for several reasons. The cell is a syncytium of very large dimensions, and each electroplax is enclosed in a compartment (FIGURE 1). At the level of the organ of Sachs the compartments are more or less rectangular in shape and their dimensions vary between 4 and 15 mm. in length, 0.5 and 1.5 mm. in height, and 0.5 to 1.5 mm. in thickness; the extracellular space occupies as much as 95 per cent of the compartment. A particularly interesting feature is the fact that the cell is formed by 2 opposite faces that differ both in function and in structure. The caudal face is an innervated and conducting membrane and contains many synapses (1 every 5 to 30 μ), while the opposite face is nonconducting. The nonconducting face has many digitations. Recent electron micrographs show, however, that fingerlike invaginations are found all over the cell, but the density and size of such formations are higher at the noninnervated membrane. The intracellular reticular formations seem to be located essentially in the vicinity of the nonconducting membrane.

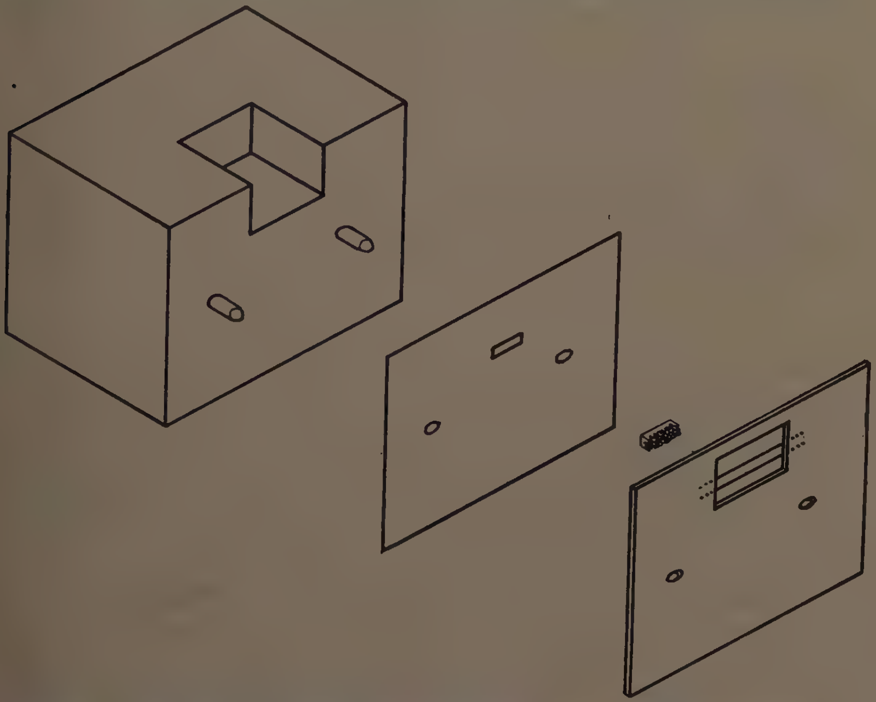

FIGURE 2. Schematic presentation of the arrangement using a single isolated electroplax to separate 2 pools of fluid; it permits study of separate properties of the conducting and nonconducting membranes. The drawing shows the respective positions of one chamber for the pool of fluid, the sheet of nylon containing a window, the single electroplax, and the grid used to press the cell against the window. The other chamber is not shown in the drawing.

The large dimensions of the electroplax made it possible to isolate one single cell by dissection and to mount it between two pools of fluid (FIGURE 2). If one takes the precaution of removing completely the connective tissue belonging to the adjacent compartment, the bypassing of ions can be avoided and any exchange of ions between the two pools of fluid must go through the cell.

An interesting aspect of the preparation is the possibility of studying the properties of both membranes separately. Indeed, one of the results presented in this paper is the demonstration that the innervated and noninnervated membrane, which differ quite considerably as to structural characteristics, behave differently also as to functional behavior.

Studies with single isolated electroplax of the electric eel and the skate were initiated about 3 years ago, and the methods and results have been presented in preceding papers.[3-7] In the present communication more data about the flux of cations in various conditions and new facts about the effect of the ionic environment on the resting potential will be presented. The results are far from being complete. Electric eels of the large size required are not always readily available in the city of New York, and the many necessary experimental conditions such as, for instance, the availability of radioactive ions, are not always simultaneously fulfilled. Nevertheless, the results obtained to date offer new facts that may be of interest to those engaged in permeability studies and therefore may justify this presentation.

METHODS

A single electroplax is isolated from the bundle of Sachs of *Electrophorus electricus* L. and mounted between 2 chambers in such a way that the cell separates 2 pools of fluid, one of them bathing the innervated membrane and the other the noninnervated one. Details of the method have been described previously,[4-5] including the mathematical treatment leading to the calculation of the flux. A correcting factor was calculated according to Keynes,[8] taking a value of 0.95 for the extracellular space. This correcting factor has always been found to be very near unity for both Na and K. The dimensions of the extracellular space vary over a wide range, not only because the amount of ground substance left during the dissection attached to the cell is variable, but also because of variations in the dimensions of the cell, as can be appreciated during the dissection.

To measure the influx of an ion species across the innervated membrane, the isolated cell is mounted in a device similar in principle to the one described;[4] however, the volume of the pools is reduced to 1 ml. The radioactive saline is kept in contact with the innervated membrane for a short period of time, generally 10 min. The cell is then mounted before a Geiger tube and washed in a stream of inactive saline, and the radioactivity remaining in the cell is then measured. When plotted on a semilogarithmic paper, the results can be analyzed and the activity present in the cell at 0 time can be found by extrapolation. If the specific activity of the bathing solution is known, and if the loss of activity from the cell during the short period of incubation is assumed to be negligible, the influx can be easily calculated. The influx of ions across the noninnervated membrane cannot be measured with reliability by this method. Because of the presence of innumerable digitations it is impossible to remove the connective tissue surrounding this membrane. This connective tissue makes up almost the entire thickness of the preparation and is an obstacle to the diffusion of the ions. Since the exact thickness of this connective tissue layer is difficult to estimate, no attempt has been made to measure the influx across the noninnervated membrane and to correct the value obtained for the delay brought about by the diffusion effect.[6]

The outflux of ions across both innervated and noninnervated membranes is measured as follows. A cell is first soaked in a radioactive saline. The cell is then mounted between 2 chambers in the usual way. The volume of each chamber is 1 ml., and the top of each chamber is closed by means of a screw cover made of Plexiglas. Each such cover has a set of silver wires dipping in the solution and acting as stimulating and recording electrodes (FIGURE 3). Inactive saline passes through both chambers, and the fluid coming out of them is collected separately. The radioactivity is measured in an immersion tube. The activity coming out of the cell is measured under these conditions. Knowing the specific activity of the soaking solution and assuming that after the soaking period the specific activity of the cell is equal to that of the soaking solution, the outflux across the innervated and noninnervated membranes can be calculated.[5] The intracellular concentration of the ion studied also can be estimated.

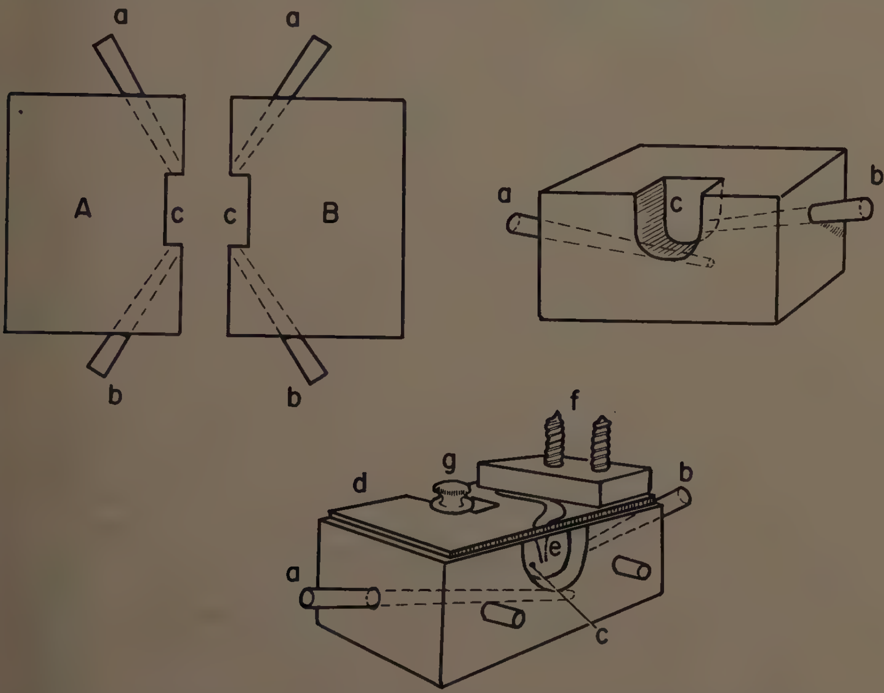

FIGURE 3. Device used to measure outflux across innervated and noninnervated membrane separately. Upper left: *A* and *B*, chambers with pools *c*, *a*, and *b*: inlets and outlets for the washing fluid. Upper right: three-quarter view showing how inlet and outlet are connected with the pool. Below: three-quarter view showing how the cover *d* is screwed in *g* on top of a chamber; *e* are electrodes (stimulating and recording) dipping in the pool *c*; *f* indicates the contacts used to connect the electrodes with the stimulating and recording equipment.

The measurement of the fluxes across innervated and noninnervated membranes requires large specimens about 1 m. in length in order to obtain cells of regular shape and at least 1 mm. in width. Frequently considerable difficulties were encountered in obtaining cells of good size. If the cells were not satisfactory, the total outflux of an ion was then measured and calculated from the rate of exchange of the intracellular ion species in the manner described previously.[5]

The presence of O_2 is critical, so the saline must be thoroughly gassed with pure O_2. Great care must be taken when the cells are stored, and the mechanical disturbances caused by the bubbling of the gas must be reduced to a minimum; otherwise, the cells may be irreversibly damaged. In order to demonstrate the importance of an adequate oxygen supply, Na_i and K_i have been measured in various conditions. During anoxia, it was found that the Na_i increases, while K_i decreases, and the magnitude of the action potentials varies accordingly. For instance, it was found in one case that if the cells were dissected in a freshly prepared saline without O_2, the action potential was 118 mV. Oxygenation of the saline brought the magnitude of the action potential to 133 mV in 20 min. This decrease of the action potential during anoxia might be explained either by the change in the ionic distribution, by a direct effect on the mechanism generating the action potential, or by a combination of both factors.

As mentioned previously, the cells are dissected from specimens from which one slice is cut every day. A single specimen provided an adequate supply for one week. It has been noted that a small decrease in the magnitude of the action potential takes place at the end of the week, but generally, after thorough oxygenation, the action potential again reached the value that had been found on the first day. For instance, in one observation the action potential on the first day turned out to be 118 mV; 8 days later it was 88 mV without oxygen. After 20 min. of oxygenation the magnitude of the action potential was 100 mV; after 25 min, 106 mV; and, after 95 min., 112 mV. As will be seen below, the flux values are also modified. By cutting a slice of electric tissue from the specimen every day, the vascularization of the remaining part of the electric organ is apparently impaired, since the 2 principal blood vessels are cut. Furthermore, there is always development of an edema in the neighborhood of the cauterized section. Consequently, the oxygen supply of the most distal part of the electric organ must be impaired; this may explain the determination observed.

The area of the cell is measured with a micrometric ocular piece, and the electric measurements were done using either external or internal electrodes. Electric activity was observed on a cathode-ray oscilloscope with photographic attachment, and the resting potential was recorded on a graphic recorder.

The saline used has the following proportional composition (in μM/ml.): NaCl, 160; KCl, 5; $CaCl_2$, 6; $MgCl_2$, 1.5; and succinate-Na_2, 10. The *p*H of the saline is 7.2. In the experiments in which Na-free saline was used, NaCl was replaced isoosmotically by sucrose. Succinate-Na_2 is then replaced by K-phosphate buffer (*p*H 7.2). Wherever the concentration of K was increased a corresponding amount of Na was withdrawn.

RESULTS

The Flux of Cations at Rest

The flux values for Na and K across both innervated and noninnervated membranes are shown in TABLE 1. For reasons explained, above under METHODS, no attempt has been made to measure the influx across the noninnervated membrane. The data indicate that the flux of both Na and K across the innervated membrane is of the same order of magnitude, the values for K being slightly smaller.

The outflux of Na and K across the noninnervated membrane is significantly higher than the corresponding values for the innervated membrane. In this case the values for K are markedly smaller. The values for the outflux of both ions across the innervated membrane are higher than the influx values. This may be due to the fact that the outflux experiments were always done one day after the influx determination. It was indeed found that in the same specimen the flux values varied according to the time elapsed since the first dissection. TABLE 2 shows the flux values measured on the same eel at 10-day intervals to illustrate the marked difference.

TABLE 1

FLUX OF NA AND K ACROSS THE MEMBRANES OF A SINGLE ISOLATED ELECTROPLAX OF *ELECTROPHORUS ELECTRICUS* L. (RESTING CONDITIONS)
(Flux Values Are Given in μM/cm.^{2}/min.)

Ion species	No. of observ.	Innervated membrane			Noninnervated membrane		
		Mean value	S.D.*	S.E.†	Mean value	S.D.*	S.E.†
Influx							
Na	8	*0.0016*	0.00067	0.00024			
K	6	*0.0011*	0.00061	0.00025			
Outflux							
Na	4	*0.0038*	0.0020	0.0010	*0.054*	0.042	0.021
K	4	*0.0020*	0.00071	0.00036	*0.0077*	0.0014	0.0007

* S.D. = standard dev. of each observation.
† S.E. = standard error of mean.

Effect of the Ionic Environment on the Cation Fluxes and on the Resting Potential

Na outflux in K-free medium. In preliminary experiments, the total outflux of Na has been measured in terms of the rate at which labeled Na leaves the cell. A cell is equilibrated in a radioactive saline and is then mounted before a Geiger tube and the activity washed out in a stream of inactive saline. In that procedure, the activity remaining in the cell is measured. Obviously, this value is related directly to the rate of escape of the radioactive isotope from the cell.[5] FIGURE 4 shows that in a K-free saline the outflux of Na decreases;

that is, the rate of loss of activity from the cell decreases. This is a reversible phenomenon, as may be seen after return to normal conditions. TABLE 3 gives the data of a typical experiment in which Na outflux has been measured separately across both innervated and noninnervated membrane. In the

TABLE 2
OUTFLUX OF NA AND K ACROSS INNERVATED AND NONINNERVATED MEMBRANES OF A SINGLE ISOLATED ELECTROPLAX MEASURED ON THE SAME EEL AT 10-DAY INTERVAL
(Flux in μM/cm.^{2}/min.)

	Outflux		
	Innervated	Noninnervated	Distance*
Na a†	0.0025	0.02	1.7
b‡	0.04	0.56	1.5
K a†	0.003	0.0063	1.4
b‡	0.033	0.064	1.2

* Approximate distance in meters from the rostral end.
† First day.
‡ Tenth day.

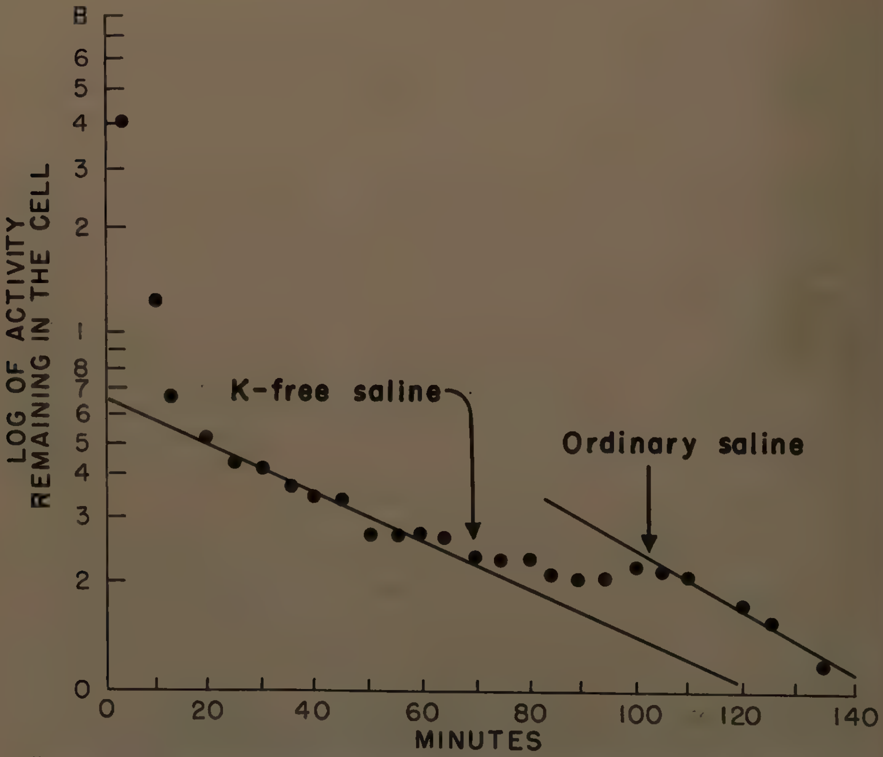

FIGURE 4. Rate of exchange of intracellular Na in the presence of a K-free saline. The activity remaining in the cell (log scale) is expressed in cpm/min. as a function of time (in minutes).

absence of K, the outflux of Na across both membranes is reduced. The effect is reversible.

Na outflux in Na-free medium. FIGURE 5 shows the results of an experiment in which the total outflux of Na can be estimated from the rate of exchange of intracellular Na. In the absence of Na in the washing fluid, the rate of exchange of intracellular Na increases. In a preliminary experiment reported elsewhere,[5] the outflux of Na was found to decrease in a saline containing 20

TABLE 3
EFFECT OF K-FREE SALINE ON THE OUTFLUX OF NA ACROSS THE INNERVATED AND NONINNERVATED MEMBRANE OF A SINGLE ISOLATED ELECTROPLAX
(Flux in μM/cm.^{2}/min.)

	Outflux	
Type of saline	Innervated	Noninnervated
Standard	0.0072	0.127
K-free for 20 min.	0.0030*	0.018
Returned to standard:		
after 10 min.	0.0065†	0.051
after 40 min.	0.0070†	0.130

* Mean of 20 min.
† Mean of 10 min.

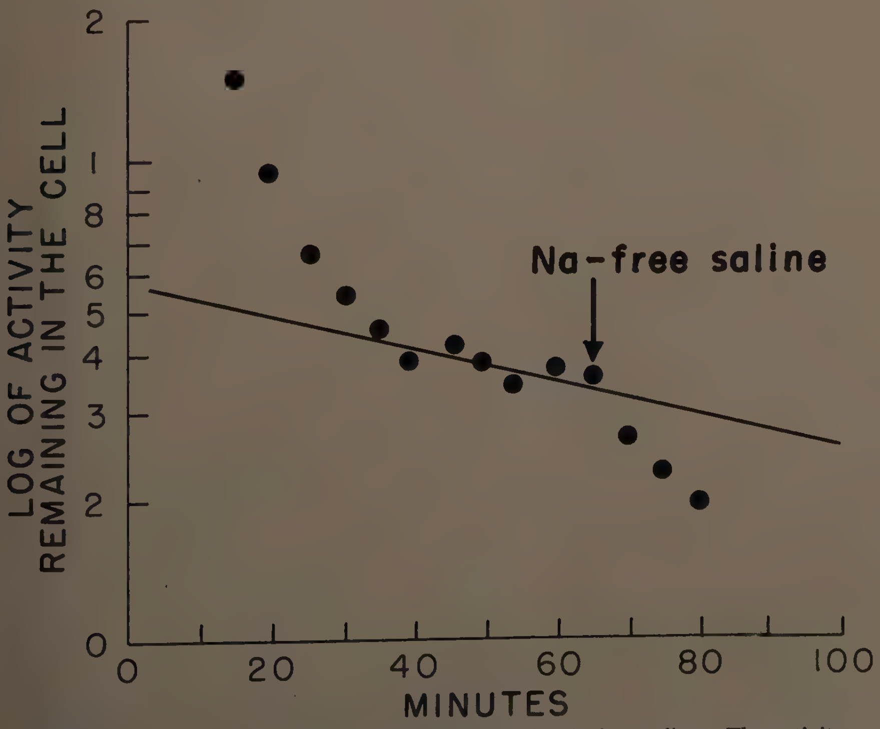

FIGURE 5. Rate of exchange of intracellular Na in Na-free saline. The activity remaining in the cell is expressed in cpm/min. on a log scale, as a function of time (in minutes).

mM/ml. of Na (normal value: 180 mM); in a Na-free saline the cell becomes slowly depolarized (unpublished experiments). The two results are therefore not necessarily in contradiction.

K outflux in Na-free medium. K outflux was measured in terms of the rate of exchange of intracellular K. It was found that if the NaCl is replaced osmotically by sucrose, no detectable change in the rate of exchange of K could be noticed. This method of estimating the change in outflux is less sensitive than that consisting of the measurement of the activity leaving the cell; the results do not exclude the fact that the K outflux could be affected slightly by a Na-free saline (FIGURE 6).

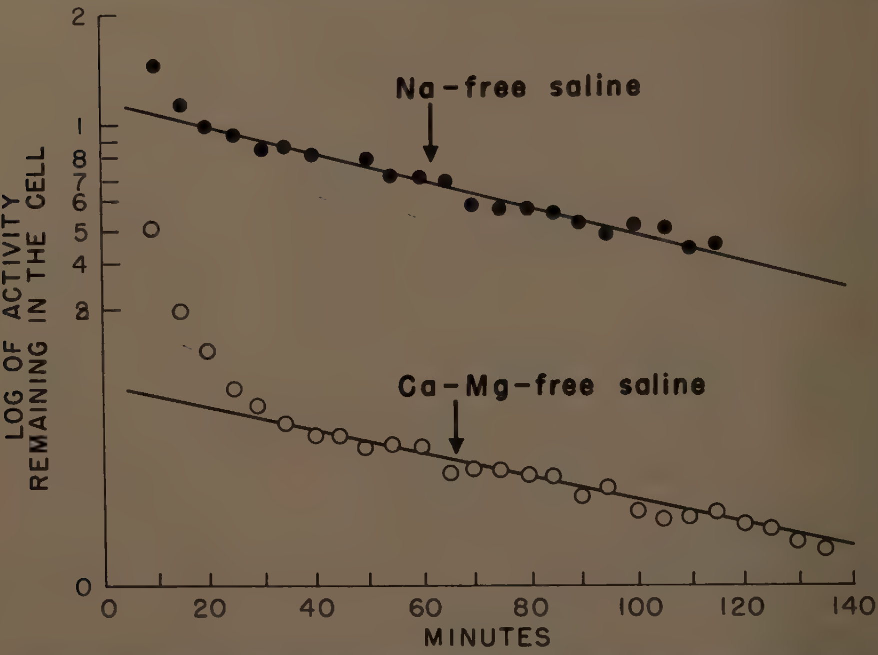

FIGURE 6. Rate of exchange of intracellular K in Na-free and Ca-Mg-free medium. The activity remaining in the cell is expressed in cpm/min. on a log scale as a function of time (in minutes).

K outflux in a Ca-Mg-free saline. The outflux of K, estimated from the curve relating the amount of activity remaining in the cell as a function of time, seems to be unaffected by the absence of Ca and Mg in the saline (FIGURE 6).

The resting potential. The potential difference measured across the innervated membrane is insensitive to the outside K concentration.[6] Since the resting potential is unaffected by 2,4-dinitrophenol (unpublished results), this rules out the active transport of Na out of the cell as the direct origin of the resting potential. On the basis of experiments with a saline in which Cl was replaced by SO_4, it was concluded that the resting potential could not be a diffusion potential for Cl. This view must be revised in the light of new developments. The saline used contained 6 mM Ca/l. If the Ca content is lowered to 1 mM, then a tenfold change in the outside concentration of K brings about a change of 35 mV in the potential difference existing across the innervated membrane. On the other hand, if the Ca content of the solution bathing the noninnervated membrane is increased to 12 mM, a tenfold change in the outside concentration of K in the solution in contact with the noninnervated membrane still brings about a change of about 35 mV in the potential difference existing across the noninnervated membrane (FIGURE 7). On the other hand,

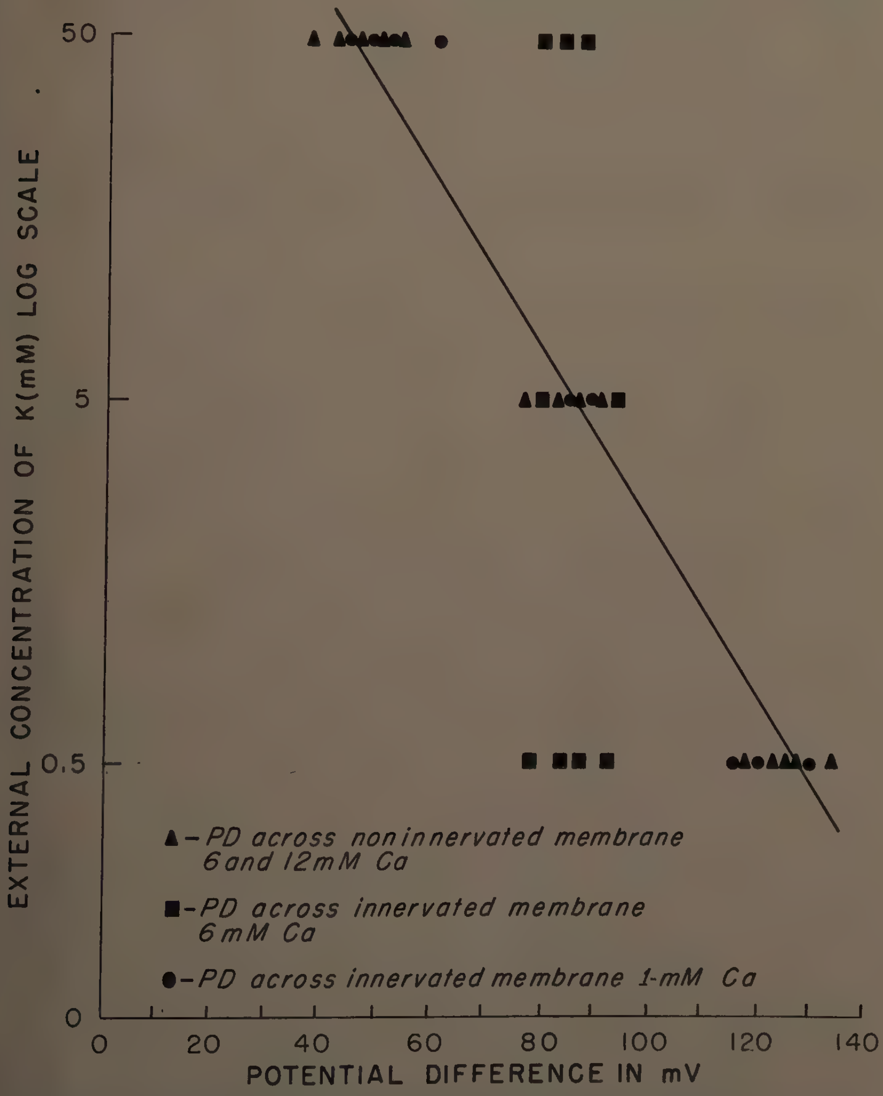

FIGURE 7. Resting potential across innervated and noninnervated membranes as a function of K and Ca concentration in the outside medium. Ordinate, K concentration in mM/ml. on a log scale; abscissa, potential difference in millivolts.

too, the K permeability of the innervated membrane is strongly dependent on the Ca content of the bathing solution; at high calcium concentration the permeability to K is low, and the contribution of the gradient of K to the potential difference becomes negligible. What, then, may be the origin of the resting potential at a high Ca concentration? The experiments on the effect of SO_4 reported in the preliminary note do not exclude Cl as being the source of the potential difference across the innervated membrane, since a part of the Ca could have been precipitated as $CaSO_4$. If this is true, the membrane may behave in SO_4 saline as in a low Ca saline, that is, as a K electrode.

Effects of Some Antimetabolites on the Flux of Cations

Carbamylcholine. As has been shown previously, carbamylcholine, a quaternary ammonium derivative, acts exclusively at the postsynaptic membrane where it competes with acetylcholine for the receptor protein.[4] It is a depolarizing agent, which accounts for its secondary effect on the electric response elicited directly. The influx of Na across the innervated membrane is increased by carbamylcholine, as may be seen from the figures of TABLE 4. Thus, the results clearly indicate that carbamylcholine increases the permeability of the postsynaptic membrane to Na, consistent with the fact that carbamylcholine is a depolarizing agent. When measured in terms of intracellular exchange, the outflux of K and Na are not affected appreciably by carbamylcholine.

TABLE 4

EFFECT OF CARBAMYLCHOLINE UPON THE INFLUX OF NA ACROSS THE INNERVATED MEMBRANE OF A SINGLE ISOLATED ELECTROPLAX

(Flux in μM/cm.^{2}/min.)

Expt. No.	C*	E†
1	0.018	0.027
2	0.0013	0.0114
3	0.0022	0.0081
4	0.0017	0.0051

* C: control, influx during 10 min. of contact with Na^{24}.

† E: experiment, influx during 10 min. of contact with Na^{24} and 25 μg./ml. carbamylcholine.

d-Tubocurarine chloride. In the presence of curare, the spike elicited by neural stimulation is abolished. Even at such a high concentration as 5 mg./ml., curare does not affect the response of the electroplax to a direct stimulation. This has been explained in terms of a permeability barrier for compounds insoluble in lipids, such as monoquaternary and diquaternary nitrogen derivatives. If curare is applied to both innervated and noninnervated membranes, the outflux of K is reduced across both membranes. As shown in TABLE 5, the outflux across the innervated membrane is less affected than that across the noninnervated one. As could be expected from these results, the rate of exchange of intracellular K is reduced by curare. The rate of exchange of intracellular Na does not seem to be affected by curare, while the influx of Na measured across the innervated membrane appears to be decreased. However, due to the individual variations, more data must be treated statistically in order to express quantitatively the extent of the reduction in Na influx.

TABLE 5
EFFECT OF *D*-TUBOCURARINE UPON THE OUTFLUX OF K ACROSS THE INNERVATED AND THE NONINNERVATED MEMBRANE OF A SINGLE ISOLATED ELECTROPLAX
(Flux in μM/cm.^{2}/min.)

	Outflux	
Expt. No.	Innervated membrane	Noninnervated membrane
1 C*	0.0025	0.0103
E†	0.0020	0.0036
2 C	0.0022	0.0068
E	0.0018	0.0024

* C: control.
† E: experiment, 100 μg./ml. *d*-tubocurarine chloride in the bathing fluid.

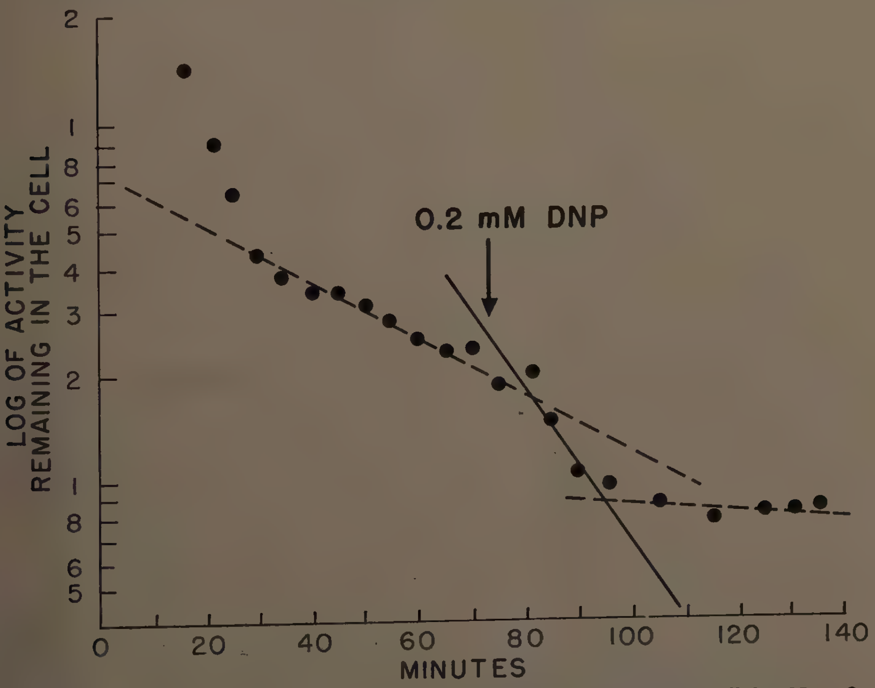

FIGURE 8. Effect of DNP (0.2 mM) on the rate of exchange of intracellular Na. Ordinate: activity remaining in the cell in cpm/min. Abscissa: time in minutes.

Eserine. At the concentration of 1 mg./ml., eserine blocks both direct and indirect spike within 15 min. At this concentration, the rate of exchange of intracellular Na was not affected.

2,4-Dinitrophenol (DNP). In the presence of a 0.2 millimolar solution of DNP (*p*H 7.2) the rate of exchange of intracellular Na seems in the initial phase to be increased, but later it decreases, as shown in FIGURE 8.

The Flux of Na and K During Activity

In a few experiments, the flux of Na and K has been measured in order to see if, qualitatively, the ionic movements in the eel electroplax during activity are of the same nature as in other conducting tissues studied thus far. TABLE 6 shows some typical results.

The Na influx across the innervated membrane is increased during a stimulation at a frequency of 5 per second. Expressed in terms of net amount of Na entering the electroplax, the figure obtained is about 0.9 $\mu\mu$M/cm.^{2}/impulse. In other experiments, the values found were of the same order. As far as the K is concerned, it can be seen that the outflux is increased across both membranes during activity and that the increase is much more important across the noninnervated membrane than across the innervated one. This is what one should have expected in considering the physiology of the electric organ. Since K is the most important intracellular cation, and since during activity current is flowing across the noninnervated membrane, the outflux of K should be increased across this structure during activity.

TABLE 6

FLUX OF NA AND K ACROSS THE MEMBRANES OF A SINGLE ISOLATED ELECTROPLAX DURING ACTIVITY

(Flux in μM/cm.^{2}/min.)

	Innervated	Noninnervated
	Influx	
Na C*	0.00047	
E†	0.00073	
	Outflux	
K C*	0.032	0.064
E†	0.049	0.192

* C: control.

† E: experiment, cell stimulated for 10 min. at a frequency of 5/sec.

The data obtained thus far do not yet make it possible to relate in a quantitative way the outflux of K, the influx of Na, and the amount of current drawn through the preparation during activity, because the permeability of the cells varied markedly, as shown by the control values (see above under METHODS). Many more determinations are therefore necessary for a really quantitative picture of the respective rates in relation to activity.

DISCUSSION

The Driving Force Responsible for the Movement of Cations

Influx and outflux of Na across the innervated membrane are of the same order of magnitude, although the outflux values seem to be slightly higher than the influx values. More results are necessary, however, to see whether or not the difference is statistically significant. The influx experiments were generally done at the beginning of the week, in order to work with the highest possible specific activity of Na. Consequently, the cells were isolated from the first slice cut from the specimen. The better physiological condition of the electroplax of this first slice may explain the difference observed. It is possible, indeed, that later the impairment of the vascularization may be such that an anoxia produces metabolic lesions in the most distal part of the electric organ (see under METHODS, and TABLE 2). Another possibility may be a higher flux rate in the proximal than in the distal part of the Sachs organ, although this appears unlikely. In any case, there is no difference in potential between the solutions bathing the innervated and the noninnervated membrane separately. Consequently, it seems reasonable to assume that there is no net flux of Na across the electroplax; that is, that under physiological conditions influx and outflux across the innervated membrane are equal.

The K fluxes across the innervated membrane in both directions are of the same order of magnitude. Again, the outflux values are higher but, as in the case of Na, it could be that the flux values increase due to the deterioration taking place with the greater number of slices cut from the specimen.

TABLE 7
COMPARISON OF CALCULATED AND EXPERIMENTAL VALUES FOR THE FLUX RATIO OF NA AND K
(Potential Difference = 90 mV)

	Concentration, μM/ml.		Flux ratio, M_{out}/M_{in}	
	In	Out	Calc.	Expt.
Na	10	180	1/560	ca. 1
K	170	5	1.009	ca. 1

The outflux of both Na and K is higher across the noninnervated than across the innervated membrane. This is consistent with the resistance measurements since it has been found that the resistance of the innervated membrane is about 5 ohm/cm.^{2} against a value of 0.5 ohm/cm.^{2} for the noninnervated membrane.[9, 10]

The influx of cations was not measured across the noninnervated membrane for the reasons given under METHODS, but there is actually no good reason to believe that the intracellular concentration of either K or Na is not in a steady state. As a consequence, we must then assume for each cation a flux ratio of 1 at the noninnervated membrane.

The forces involved in the movement of Na and K through the membranes of the electroplax may be analyzed if we know, besides the flux values, the ion concentrations inside and outside the cell and the potential difference (PD) existing across the membrane. The (Na_i) has been found to vary between 5 to 23 μM/ml.[2] while the (K_i) is about 170 μM/ml. (unpublished results). The PD, measured with intracellular microelectrodes, varies between 80 and 90 mV, the inside of the cell being negative with respect to the outside. If the cations are moving passively, that is, along the electrochemical gradient, the flux ratio found experimentally should be equal to the value predicted according to the gradient of electrochemical potential.[5] TABLE 7 gives the flux ratio calculated according to the electrochemical gradient and the values found experimentally. In the case of Na, the flux ratio found experimentally is higher than the one calculated. This clearly indicates that Na is actively transported out of the cell. Energy derived from metabolism is needed to perform this work. Indeed, if DNP is applied, the outflux of Na is inhibited. Prior to the inhibition, there is an increased outflux for which we are unable to offer any explanation at the present time. The flux ratio calculated for K and that found experimentally are very close to each other, which means that it is not necessary to postulate the existence of an active transport to explain the unequal distribution of K across the electroplax membrane.

FIGURE 2 and TABLE 3 show that influx of K and outflux of Na seem to be dependent on each other. The best interpretation thus far may be the assumption of an exchange of Na for K when Na moves out of the cell by active transport. Under those conditions, there is no net transfer of positive charge across the membrane; the active transport of Na cannot then be considered to be responsible for the generation of a PD in the manner first described by Ussing and Zerahn in 1951.[11] As in some other tissues studied thus far (such as giant axon and frog skin) active transport is not electrogenic,[12, 13] and the source of the PD existing across the membrane of the electroplax must be explained in a different way.

The outflux of Na is increased in Na-free saline. This is at variance with what was found when the outflux was measured in saline containing 20 μM of Na/ml.; in the latter case the outflux was found to be decreased. This result was interpreted as an argument in favor of an exchange reaction for Na going on across the membrane of the electroplax. This is not necessarily in contradiction to the results reported here. It has been found, indeed, that, in the absence of NaCl, a slow depolarization takes place, probably due to the removal of most of the Cl in the outside medium (unpublished result). The increased outflux of Na in a sucrose saline could then be due either to the decrease in electrochemical potential or to an increase in the permeability of the membrane due to the alteration of the ionic strength. Since the outflux of K does not seem to be appreciably affected by sucrose saline, the first suggestion would seem the better. Theoretically, however, the decrease in electric potential should also lead to an increase of K outflux. Since the permeability to K is low in the presence of high Ca, it may be that the possibly increased outflux cannot be detected by the method used (rate of exchange of intracellular K measured as the activity remaining in the cell).

These considerations apply, certainly, to the results expressing the influence of Ca- and Mg-free saline on the K outflux (FIGURE 6). The study of the effect of Ca and K on the resting potential shows that the permeability of the innervated membrane to K is strongly dependent on the presence of Ca (FIGURE 7). However, when the outflux of K is measured in terms of rate of exchange, the activity remaining in the cell appears to be affected in an appreciable way only by important modifications of outflux. Since the outflux of K across the innervated membrane is only a fraction of the total outflux, the outflux of K in Ca- and Mg-free saline should be measured across both innervated and noninnervated membranes separately, in terms of the activity coming out of the cell, in order to measure the actual effect.

The PD existing across the innervated membrane is insensitive to both DNP (unpublished results) and to outside K concentration (FIGURE 7). These observations speak against both active transport of Na and the unequal distribution of K as the source of the PD observed. Despite the fact that a SO_4 saline does not affect the PD, it is now believed that at high Ca concentration the PD measured across the innervated membrane is essentially a diffusion potential for Cl. As explained above, it is assumed that insufficient precautions were taken in the use of SO_4 instead of Cl to ensure the proper concentration of Ca. As shown in FIGURE 7, at low Ca concentration (1 mM/ml.), the PD existing across the innervated membrane is dependent on the gradient of K. This might have been the case in the SO_4 medium.

The Flux of Cations During Stimulation

According to the results presented, there does not seem to be any qualitative difference between the electroplax and other conducting tissues as far as the ionic basis of the action potential is concerned. In the electroplax, electric activity is accompanied by a decrease in resistance[9, 10] and an increase in both Na influx and K outflux (TABLE 6).

It is interesting to consider the behavior of K across the noninnervated membrane. Since during a discharge an electric current is flowing across the whole cell, it must be carried across the noninnervated membrane either by an anion flowing in or by a cation flowing out of it. The results shown in TABLE 6 show that intracellular K contributes to the current carried through the noninnervated membrane. Since no experiment was done with Cl, it is actually impossible to define how much of the current is carried by K.

Many more experiments are needed to analyze in detail the quantitative relationship between Na influx, K outflux, and the amount of current drawn through the preparation. The results obtained so far are satisfactory, in the sense that they show that qualitatively the electroplax behaves like any other conducting tissue.

However, one of the most important achievements is the demonstration that the preparation developed may be used for measuring ion fluxes across the membranes of the electroplax, whereas the pieces of electric tissue used until 1955 did not permit this type of study because of the complexity of their preparation.

Effects of Antimetabolites on the Ion Flux

It seems actually well established that a chemical reaction is involved in the permeability change responsible for the action potential. Besides all the pharmacological and biochemical evidence accumulated to date, the recent measurements of heat production by Hill and his associates[2] are conclusive. The temperature coefficient of the process and its energy of activation are also outside of the range of a purely physical process.[3] The only theory so far put forward to integrate the vast amount of observations on the subject has been proposed by Nachmansohn.[1] In his theory this author attributes a fundamental role to acetylcholine in the generation of the action potential. In view of the effect of some inhibitors of anticholinesterase on ion movements in various tissues, many authors have proposed that the cholinesterase system could play a role also in the active transport of Na across living membranes. Ionic movements are indeed affected by cholinesterase inhibitors in structures such as the gill of the crab,[14] frog skin,[15] red blood corpuscles,[16] and muscle.[17] The mechanism through which active transport of Na is performed is not as simple as was believed after the first experiments of Ussing.[18] The possibility of an exchange between Na and K, the origin of the PD, the role of Ca, and other observations suggest that, by affecting one of the parameters to the exclusion of the others, one may throw the entire system out of balance. In the light of the results presented above, it appears questionable whether, for instance, curare affects ion movements in frog skin by direct interference with the mechanism of exchange between Na and K, or whether, as seems to be more likely, only the passive movements of Na and K are affected by curare (K outflux and Na influx).

As far as the conducting tissue is concerned, the effect on the cation fluxes of substances known to interfere with the acetylcholine system are consistent with the idea of a primordial role of this system in the generation of the action potential. Indeed, carbamylcholine increases the Na influx.

In the presence of curare, the decrease in K outflux across the innervated membrane raises the important question of its site of action. The innervated membrane of the eel is a complex structure formed by synapses and conducting membrane. On the basis of histological evaluation, the area of the postsynaptic regions represents not more than 10 per cent of the total surface area of the innervated membrane. As shown in TABLE 5, the reduction of K outflux brought about by curare is about 15 per cent for the innervated membrane and 65 per cent for the noninnervated one. It is tempting to assume that the effect of curare on the K outflux across the innervated membrane is essentially located at the postsynaptic regions. This interpretation is supported by the absence of an effect of curare on the spike elicited directly. Contrary to the measurements of the outflux, the influx determinations were carried out on two different cells: one for the control and one for the experiment. In view of the individual variations, more results are necessary for evaluating the extent of the reduction of the influx of Na effected by curare. In the light of the results with K, it may be tentatively assumed that curare affects only the influx of Na in the postsynaptic areas of the innervated membrane. Thus, two compounds known to interfere with the electric activity of the electroplax affect the cation fluxes in a way that might have been predicted from Nachmansohn's theory. The receptor activator (carbamylcholine, for instance) mimics the physiological effect of a release of acetylcholine; such drugs induce a structural change at a strategic level by their action on the receptor protein, increasing the permeability to Na; hereby a depolarization takes place. Conversely, the receptor inhibitors (curare, for instance) combine at the same strategic level (that is, with the receptor protein), but for some not yet fully understood reason of complementarity, they do not induce the structural change leading to an increased permeability. The new aspect brought to light by the present results, then, is the reduced permeability to Na and K by a receptor inhibitor such as curare.

The lack of effect of the quaternary ammonium derivatives on the electric phenomena in conducting tissue has been explained on the basis of their lipid insolubility.[19, 20] Recently, in this laboratory we have synthesized quaternary ammonium derivatives that are soluble in lipids. With these compounds an effect on different conducting tissue has been shown.[21] In some preliminary experiments, the flux of Na and that of K has been studied in the presence of those lipid-soluble derivatives. Both influx and outflux of Na and K seemed to have first decreased. If the contact is prolonged, the flux increases. The effects obtained depend, however, largely on the conditions of application, such as, for example, Ca concentration. In the beginning these conditions were not well defined, but they are better understood now that they have been explored with various types of conducting tissues and by modifying several factors.[22-25]

Energy supplied from phosphorylated compounds is believed to be necessary, either directly or indirectly, for active transport of Na out of the cell. DNP blocks oxidative phosphorylation and has been found to inhibit the active transport of Na in various tissues.[12, 26] We are unable to find an explanation for the initial increase observed in the electroplax.

The Functional Structure of the Conducting and Nonconducting Membranes

The ultimate aim of the permeability studies is to acquire information about the organization and the chemical nature of the structures responsible for the unequal distribution of ions observed between a cell or an organism and its surrounding medium. This problem is especially fascinating in the case of conducting tissue where a structure acquired in the course of evolution enables a cell to make use of a widely spread characteristic of the living matter, for its special function, which is that of rapidly transmitting messages between distant points.

Much new information has been obtained in recent years about ultrastructure and functional properties of cell membranes. One puzzling question is to know how those different functions are organized along the surface of the cell membrane. I propose to restrict the following discussion to the organization of a few functions performed by living membranes: namely, the handling of ions; and to consider for that purpose the two types of membrane offered by the eel electroplax: conducting and nonconducting. To clarify the discussion, let us ask the following three questions: (1) Is the structural arrangement of a conducting membrane (on a molecular level), which is responsible for the properties of specific permeability of the membrane at rest (K and Cl electrode), the same as that which undergoes a change leading to the action potential (Na electrode)? (2) Is the structural arrangement of the membrane of nonconducting cells (such as those of frog skin, cornea, gastric mucosa, urinary bladder, and the noninnervated membrane of the electroplax) responsible for the properties of specific permeability (K and Cl electrode) of the same chemical nature as the corresponding one in a conducting tissue? (3) Is the same chemical structure responsible for the active transport of Na out of the cell, and for the properties of specific permeability to K and Cl? If it were the same, the active transport of Na coupled to a K exchange and the passive movement of Na, K, and Cl would be taking place at the same sites. If this structure is chemically different, or has only some chemical relationship with the one responsible for the K and Cl permeability, then we could consider the membrane as a mosaic with spots of different chemical structure, performing different functions.

Let us first discuss the possibility of an identity of the structure responsible for the specific properties of permeability at rest and during activity. Curare affects the outflux of K across both innervated and noninnervated membranes. The action potential elicited by direct stimulation is never affected by curare. This may seem to be a difficulty for assuming a basic similarity of the structural arrangements responsible for the properties of specific permeability at rest and during activity, since only one of these two membranes has the ability to generate action potentials. However, due to permeability barriers, curare affects K outflux and Na influx only at the postsynaptic regions. Therefore, the reduction in K outflux across the innervated membrane is very small and is consistent with a localized effect limited to a small portion of the surface area of the innervated membrane. If curare were to reach the site where the action potential is generated it might affect the resting and active process in a similar way. Ca affects the permeability to K across the innervated membrane, while over an even wider range of concentration it has little or no effect at all on the noninnervated one. High Ca concentration seems to switch the innervated membrane from a K to a Cl electrode, and to prolong the electric response elicited directly. This effect is also obtained with noracetylcholine 12 at low concentration.[21, 22, 25] It seems to be in favor of a certain chemical similarity, if not identity, between structural properties responsible for the action potential and those responsible for the specific permeability at rest. However, the lack of effect of Ca on the K permeability across the noninnervated membrane, at least within a range affecting strongly the innervated one, suggests some differences between structures responsible for the specific properties of permeability in a conducting and in a nonconducting tissue.

With regard to the structure responsible for the active transport of Na out of the cell, there is good experimental evidence to show that active transport and action potential are not directly related. DNP known to affect both outflux of Na and influx of K is without effect on the electric activity. Moreover, curare does not affect active transport, nor does a Ca-free or Mg-free saline.

In conclusion, we may tentatively picture a conducting membrane as formed essentially by two types of structural properties. One is responsible for the active transport of Na out of the cell in exchange for K, thus building the ionic gradient at rest and during recovery. Another structure is responsible for the specific properties of passive permeability at rest (K and Cl electrodes) and during activity (Na electrode). In resting conditions, the influx of Na would also take place through this structure.

In a nonconducting tissue, one structure is devoted to the active transport of Na, while another one may be responsible for the characteristic permeability to the passive movement of K, Na, and Cl. The structure responsible for the specific properties of passive permeability to K, Na, and Cl in both conducting and nonconducting tissue may have some chemical similarity, since the same compound (for example, curare) affects both of them. As far as the ionic regulation is concerned, we could then picture a cellular membrane as formed by two types of structural organization and as performing two types of function. One type is essentially concerned with the exchange of Na for K and requires energy to perform this work. This is the function of active transport. In this process there is no net transfer of electric charge across the membrane; at the other site, only K or Cl are able to pass. The latter site behaves like an electrode to K or Cl, with a possible leakage of Na. Due to the unequal distribution of K and Cl, and because of the almost exclusive permeability of this spot to K and Cl, a resting potential is established.

What may happen in a conducting tissue is a modification of the latter site, causing it to become permeable to Na by a chemical reaction. This site behaves like a Na electrode, and the equilibrium potential for Na is measured. This would be the basis of the action potential.

SUMMARY

A monocellular preparation that I developed during the last three years from the electric organ of *Electrophorus electricus* has been demonstrated to be adequate for measuring ion fluxes across a conducting and a nonconducting membrane. In contrast to the complex preparations used until 1955, in which pieces of electric tissue containing many compartments with one to three rows of electroplax were studied, the new preparation contains only a single isolated electroplax. This cellular unit separates two pools of fluid, the one bathing exclusively the conducting, the other the nonconducting membrane of the cell. All ions moving from one pool to the other must pass through the cellular membranes. The new preparation has eliminated many limitations and difficulties encountered previously and offers an interesting and in many respects unique tool for the study of physical and chemical factors underlying the generation of bioelectric currents with the aid of a cell highly specialized for this function.

Although the investigations with this preparation are still in an initial phase and require much more work, the following eight facts have been observed thus far.

(1) As in most cells, the concentration of Na is low inside the electroplax as compared to that in the surrounding medium. This unequal distribution appears to be the result of an active transport from the cell's interior to the outside. The process seems to be coupled with the movement of K in the opposite direction.

(2) The flux of cations is always higher across the noninnervated membrane than across the innervated membrane.

(3) The potential difference existing across the innervated membrane is at high Ca concentration (6 mM) independent of the outside K concentration.

(4) Even at high Ca concentration (12 mM), the potential difference existing across the noninnervated membrane is dependent on the outside K concentration.

(5) During activity, the Na influx across the innervated membrane increases. The outflux of K increases across both innervated and noninnervated membranes.

(6) Carbamylcholine increases the influx of Na across the innervated membrane.

(7) Curare decreases the influx of Na and the outflux of K across both types of membranes.

(8) DNP first stimulates, then inhibits, the outflux of Na.

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MOLECULAR COMPLEMENTARITY IN ANTIDOTES FOR NERVE GASES[*]

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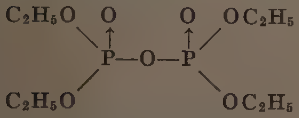
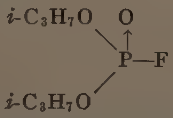
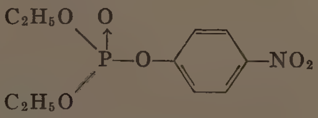
In this paper I shall describe the role of molecular complementarity in designing a compound, pyridine-2-aldoxine methiodide (2-PAM),[1, 2] which is an excellent antidote to many organophosphorus compounds that inhibit cholinesterase.[3-8] Some of these compounds are nerve gases and insecticides. Some representative toxic compounds are



Tetraethyl pyrophosphate
(TEPP)



Diisopropyl fluorophosphate
(DFP)



Diethyl *p*-nitrophenylphosphate
(Paraoxone)

The most important single feature of these compounds is the fact that they contain a phosphorus bond that is easily hydrolyzed; other aspects are relatively unimportant. The compounds can have alkyl or aryl groups, be phosphates or phosphonates, contain sulfur, selenium, or oxygen, have fluorine, chlorine, iodine, cyanide, thiocyanide or cyanate radicals, amino or substituted amino and still other groups, and all are toxic.[9]

These organophosphorus compounds, even in very low concentration, inhibit a variety of hydrolytic enzymes, but the only one of importance in acute toxicity is acetylcholinesterase, which catalyzes the hydrolysis of acetylcholine.[10, 11]

The human body contains at least two and probably several cholinesterases. Two types occur in blood; the type found in the red cells is called acetylcholinesterase, and the type found in the serum is called serum cholinesterase; these names do not denote a particular enzyme, but rather a type of enzyme. Acetylcholinesterase, in distinction to serum cholinesterase, is inhibited by high concentrations of acetylcholine and does not hydrolyze butyrylcholine or benzoylcholine, but does hydrolyze acetyl β-methylcholine.[12-15] These enzymes have no known function in blood, but in nervous tissue acetylcholinesterase plays a vital role. The alkylphosphates, when administered to an animal, pene-

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trate into nervous tissue and inhibit acetylcholinesterase, especially at junctional regions because of the greater accessibility of these sites. Junctional regions are regions of discontinuity between communicating cells such as the synapse between two nerve cells, the neuromuscular junction between a nerve ending and a muscle cell, and the junction between a nerve ending and an autonomic effector cell. These are sites where the interruption of normal function can quickly lead to death. In normal junctional function, acetylcholine is released by a nerve impulse (there is disagreement as to whether acetylcholine is released from the nerve ending and acts on the postjunctional membrane or is released in and acts in both the pre- and postjunctional membrane;[16, 17] in either case, the theory of alkylphosphate toxicity is the same). The released acetylcholine acts in the membrane to change its permeability to ions so that the membrane, which is electrically charged at rest, becomes discharged. In the case of synapses this depolarization produces an action potential in the second neuron. In the case of smooth muscle, contracture occurs. In skeletal muscle an action potential is produced in the muscle cell membrane that in turn causes contraction. The initial state of the membrane is restored by hydrolysis of the released acetylcholine. If acetylcholinesterase is inhibited, acetylcholine is not hydrolyzed but persists *in situ* and, according to the amount and location, gives rise to excitation or block. Control of function is lost.

We now consider the chemistry of acetylcholinesterase and its inhibition by alkylphosphates.

Mechanism of Hydrolysis by Acetylcholinesterase

The formal representation of the reaction of an enzyme E and a substrate S is

$$E + S \rightleftarrows E{\cdot}S \rightarrow E + \text{products}$$

We must describe the nature of the enzyme substrate compound and the hydrolytic process in which this complex is converted into products with the simultaneous regeneration of the enzyme. The whole process occurs in a few microseconds. The active site of acetylcholinesterase is made up of two principal subsites (FIGURE 1):

(1) An anionic site[18, 19] that binds and orients the cationic portion of the substrate. Coulombic forces contribute 2.0 Kcal. of binding energy (a factor of 30 in the equilibrium constant). In addition, all but one methyl group make a binding contribution of 1.2 Kcal./mole (a factor of 7). The source of this binding energy is believed to be a net van der Waals attraction between methyl groups and a hydrocarbon portion of the protein. One CH_3 group projects into the aqueous phase and thus does not contribute to binding.[20]

(2) An esteratic site $H\text{—}\underset{\cdot\cdot}{G}$, containing an essential acidic group represented by a hydrogen atom and a basic group represented by a pair of electrons. G is probably more than a single atom. Binding here is through a covalent bond between the basic group and the electrophilic carbonylcarbon atom.[19, 21]

If we compare ethyl acetate and acetylcholine, we note that a quaternary nitrogen function is lacking in the former and that, therefore, acetylcholine should be bound 30 × 7 × 7 = 1500 times better than ethyl acetate. In addition, the hydrolytic process is about 10 times more rapid for acetylcholine so that, at low substrate concentration acetylcholine, is hydrolyzed about 10^4 times more rapidly than ethyl acetate. We thus have the method by which enzymes achieve specificity; that is, by developing a structure that is molecularly complementary to a particular substrate. Such a structure allows the summation of several small interaction energies. The anionic site thus promotes the activity of the enzyme toward compounds containing a suitably located quaternary ammonium function. The hydrolytic process that occurs at the esteratic site is as follows:[22, 23]

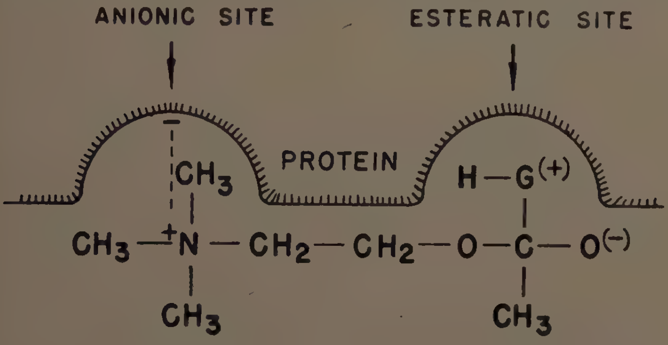
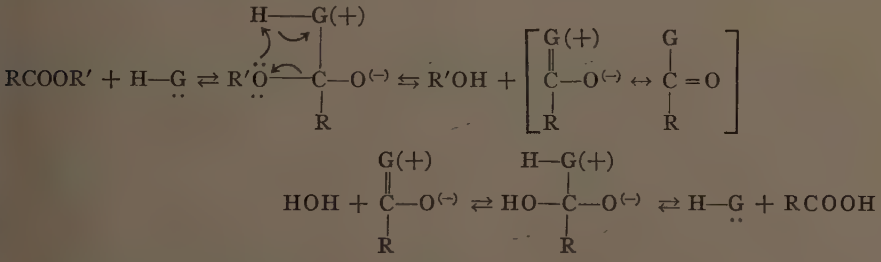


FIGURE 1. Schematic representation of the principal subsites of acetylcholinesterase.



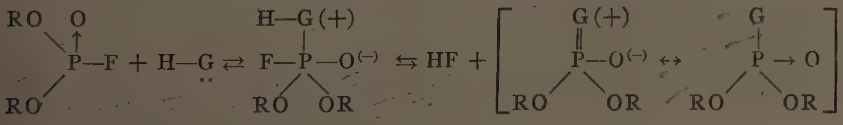
$$RCOOR' + H\text{—}\underset{\cdot\cdot}{G} \rightleftarrows R'\underset{\cdot\cdot}{\overset{\cdot\cdot}{O}}\text{—}\overset{H\text{—}G(+)}{\underset{R}{C}}\text{—}O^{(-)} \leftrightharpoons R'OH + \left[\overset{G(+)}{\underset{R}{C}}\text{—}O^{(-)} \leftrightarrow \overset{G}{\underset{R}{C}}{=}O\right]$$

$$HOH + \overset{G(+)}{\underset{R}{C}}\text{—}O^{(-)} \rightleftarrows HO\text{—}\overset{H\text{—}G(+)}{\underset{R}{C}}\text{—}O^{(-)} \rightleftarrows H\text{—}\underset{\cdot\cdot}{G} + RCOOH$$

We consider that an incipient hydrogen bond sets off an electronic cycle that results in the splitting out of an alcohol (choline, if acetylcholine is the substrate) and the formation of an acyl enzyme (acetyl enzyme, if the substrate is an acetate). The acyl enzyme then reacts with water in a few microseconds to reverse the general process and form the acid and regenerate the enzyme.

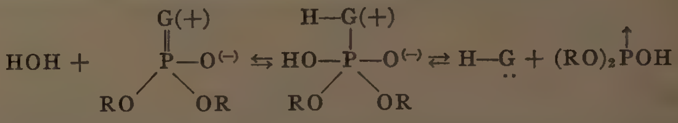
Inhibition of Cholinesterase by Organic Phosphorus Compounds

These compounds are hemisubstrates; they react as substrates to phosphorylate the esteractic site (illustrated here with a fluorophosphate),



$$(RO)_2\overset{O}{\overset{\uparrow}{P}}\text{—}F + H\text{—}\underset{\cdot\cdot}{G} \rightleftarrows F\text{—}\overset{H\text{—}G(+)}{\underset{RO\ \ OR}{P}}\text{—}O^{(-)} \leftrightharpoons HF + \left[\overset{G(+)}{\underset{RO\ \ OR}{P}}\text{—}O^{(-)} \leftrightarrow \overset{G}{\underset{RO\ \ OR}{P}} \to O\right]$$

but the next step, the hydrolysis of the phosphoryl enzyme,

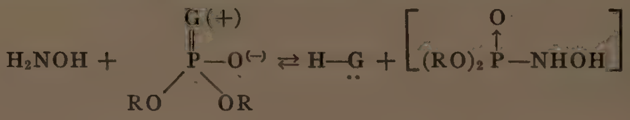


either does not occur or occurs very slowly, depending upon the nature of R. If this step were to occur rapidly, for instance, in a few milliseconds, these compounds would be substrates rather than inhibitors.[24, 25]

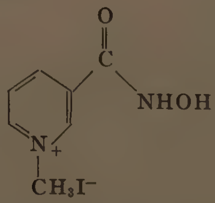
The first step with an inhibitor need not be rapid compared to the reaction of a substrate because, in contrast to a catalytic reaction in which the enzyme must react with millions of equivalents of substrate, the enzyme is inhibited completely as soon as it reacts with just one equivalent of inhibitor.

Reactivation of Inhibited Enzyme

The hydrolysis of the dialkylphosphoryl enzyme is slow, but it can be demonstrated readily in many instances by allowing the inhibited enzyme to stand in solution for several hours or days; during this period enzyme activity gradually returns. It was soon found that dephosphorylation of the inhibited enzyme with restoration of activity could be achieved much more rapidly by the addition of simple nucleophilic agents, especially hydroxylamine.[24]



This reactivation reaction can be inhibited by simple quaternary amines such as tetramethyl ammonium ion. Evidently the ammonic site is still functional (FIGURE 2). The question then arose as to whether the activity of the reactivator could be promoted by introducing a quaternary ammonium function into the reactivator, just as the hydrolysis of acetylcholine is promoted over ethyl acetate. Our first attempt in this direction, using nicotinohydroxamic acid methiodide was quite successful.



This compound was only 4 or 5 times better than hydroxylamine in reactivating diethylphosphoryl enzyme (TEPP inhibition), but it produced reasonably rapid reactivation of diisopropyl phosphoryl enzyme (DFP inhibition), which had never previously been significantly restored to activity.[26]

The hydroxamic acid is not, of course, highly nucleophilic; it is rather the conjugate base, the hydroxymate zwitterion, that is the reactivator.

We were thus greatly encouraged in our contention that a hydroxylamine derivative could be designed to exploit the binding and orienting properties of the active site and its surroundings. Evidently, what we want is a molecule that will be bound to the inhibited enzyme in such a way that the nucleophilic oxygen atom will fall one bond length from, and directed toward, the phosphorus atom. In addition, the molecule should be "rigid," that is, it should have very few and preferably only one probable configuration, so that it is necessarily bound in the correct configuration, and there will be little loss of configurational entropy.

Several hydroxamic acids were studied, and it was found that picolino hydroxamic acid was twenty times more active than nicotinohydroxamic acid methiodide.[27] The fact that aromatic rings contribute strongly to binding suggests that the ring will be bound in some one orientation. Consequently, the position of the carbon atom in the side chain is also fixed, but the nitrogen atom and the nucleophilic oxygen atom are not fixed. Rotation about the bond joining the ring to the carbon atom and about the C—N bond in the side chain generates a surface of possible oxygen positions. From the activity of this compound we judge that one of these positions is quite satisfactory.

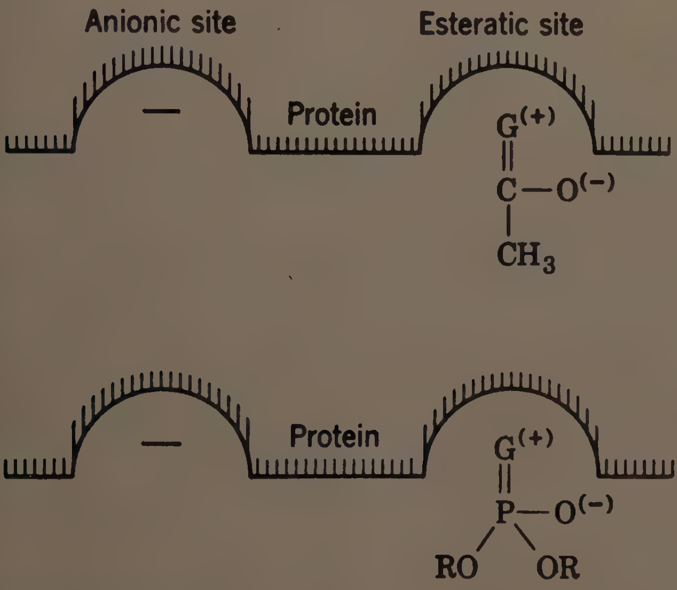
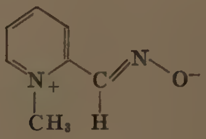


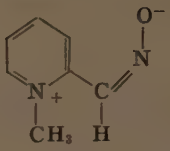
FIGURE 2. Schematic representation of the acetylated enzyme (*top*) as compared to the phosphorylated enzyme (*bottom*).

By introducing a double bond into a side chain we can greatly diminish the number of configurations and, if we have not excluded the "correct" one, we can expect, all else being equal, to obtain a more active compound. Two geometric isomers are possible, but between them they do not cover all the original positions. The odds are therefore against us, but it is just this situation that makes the return greater should we win.

The resulting compounds are the syn- and antipyridine-2-aldoxime methiodides, the conjugate bases of which are shown below.



syn



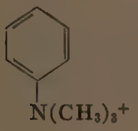
anti

The syn compound was inactive, but the anti compound proved to be amazingly active, thousands of times more active than the hydroxamic acid and almost a million times more active than hydroxylamine.

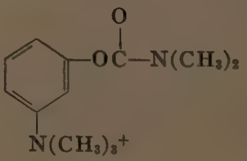
The configuration of this oxime is even more restricted than has thus far been indicated. Resonance between the oxygen atom and the ring tends to fix the oxygen atom in the plane of the ring. Of the two possible positions, the one not shown is improbable because it would involve overlap with the methyl group. There is, therefore, only one probable configuration, and evidently it is the right one.

We then undertook to confirm this conclusion by finding the position of the phosphorus atom. We proposed to do this by studying the binding of competitive reversible inhibitors.[28, 29]

It was already known from the greater binding of phenyltrimethyl ammonium ion (I), as compared to tetramethyl ammonium ion, that an aromatic ring makes a large binding contribution and is therefore very probably bound in one definite orientation. "Rigid" molecules are apt to yield the most information and, in this connection, the dimethyl carbamate of 3-hydroxyphenyl trimethyl ammonium (II) is most useful.



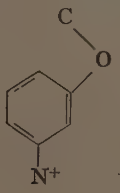
I



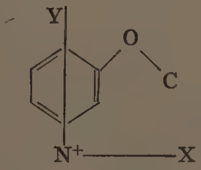
II

This compound is an extremely good inhibitor and is more than 3000 times as well bound as I. The binding contribution of the carbamate function is believed to arise largely from the formation of a covalent bond between the basic group of the esteratic site and the carbonyl carbon atom.[30] This conclusion is based upon the ease of addition of nucleophilic agents to carbonyl compounds in general and upon the correlation of electrophilic properties in a series of inhibitors with inhibitory strength. This conclusion is also supported by the formation of an acyl enzyme in the enzymic hydrolysis of substrates and by the decline of inhibition in acid media, a fact that can be explained readily by the conversion of a basic group with a pK_a of about 6.7 to its conjugate acid.[31] This is about the same value required to account for the decreased enzymic hydrolysis rate in acid solution.

Therefore, if we can locate this carbon atom we shall have the position of the carbonyl carbon atom in the enzyme substrate complex and in the acyl enzyme and, what is most pertinent for this discussion, we shall have the position of the phosphorus atom in the phosphoryl enzyme. In the molecule of II there is surely a fair amount of resonance between the phenolic oxygen atom and the ring, so that this bond has partial double bond character and the carbonyl carbon atom must lie in the plane of the ring. With this restriction there are only two relative positions possible:



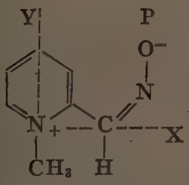
II*a*



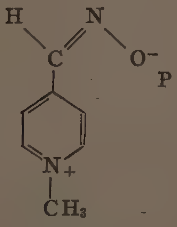
II*b*

Pyridine-2-aldoxime methiodide (anti) satisfies our theoretical requirements if disposition *b* is correct. Let us define the Cartesian coordinates based upon bond II. Take the origin as the anionic site: this is the position of the quaternary nitrogen atom. Take the *y* axis as the direction of the axis of the benzene ring and of the nitrogen to ring bond and, finally, the *x*, *y* plane as the plane of the ring. Then the coordinates of the carbonyl carbon atom in disposition *b* are 3.4, 3.3, 0 Å.

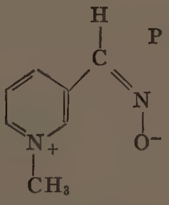
If we now place 2-PAM (anti), 3-PAM (anti), and 4-PAM (anti) and the phosphorus atom on this coordinate system we find



2-PAM (anti)



4-PAM (anti)



3-PAM (anti)

Resonance again gives us planar molecules, so that we have only 2 dispositions for each compound.* In the case of the 4 derivative, the 2 dispositions would be identical in the unbound molecule, and we therefore have only one position for the nucleophilic oxygen. In the 2 derivative, only one of the dispositions is probable, as we have already indicated. The 2 and 4 derivatives appear to be very satisfactorily oriented: the nucleophilic oxygen falls 1 bond length from and directed toward the phosphorus atom. Then not only the 2 but also the 4 derivative should be very active, and it was so found, but the 2 derivative was 40 times better, which could not be anticipated from this simple theory. Both dispositions of the 3 derivative are evidently completely unsatisfactory and the 3 derivative is inactive. These observations suggested very strongly that the *b* configuration of II was correct. However, this is what we wish to prove by a method that does not involve reactivators. We therefore undertook studies starting with 3 hydroxyphenyl trimethyl ammonium ion to explore this question further (III).

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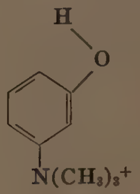
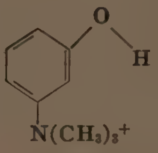
* We are not considering the dispositions obtained by rotation of 180° about the Y axis.



III*a*

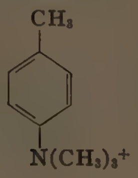


III*b*

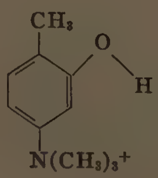
The hydroxyl group contributes a factor of 120 or 3.5 Kcal. to binding. The phenolic group lies in the plane of the ring and, again, only two dispositions are possible. These configurations are not equally probable, because the molecule is not symmetrical and, in configuration *b*, the O—H dipole is less favorably oriented relative to the electric field originating in the ammonium group. This effect is small, and it was estimated that the probability of *b* is 0.4 instead of 0.5.

The large binding contribution of the phenolic group could hardly arise from any interaction other than a hydrogen bond. This conclusion is strengthened by the fact that the binding constant decreases in alkaline solution, *p*H > 8, where the phenolic group is dissociated in contrast to the binding constants of simpler quaternary ions such as I, which do not change. Also, the 3-methoxy derivative of I is only slightly better bound than I, and about as well bound as the 3-methyl derivative.

We have seen that there is an essential basic group in the esteratic site G̣, and it would certainly be reasonable to assume that this is the group with which the hydrogen bond is formed. This group must be in the general direction of the O—H bond and about 2.7 Å from the oxygen atom. We can therefore arrive at an approximate position of G̣ if we can decide between the two configurations. This decision can be made in 2 ways. First, if III*a* is correct, introduction of a CH_3 group in the 4 position, V will prevent the hydrogen bond forming by forcing the structure into the III*b* configuration, and the binding contribution of the hydroxyl group will be lost. On the other hand, if this should be the correct configuration, the binding constant will be increased by 2 factors: (1) the intrinsic contribution of a methyl group in the 4 position, which was found to be a factor of 1.25 from the comparison 4-methylphenyl trimethyl ammonium ion IV with I; and (2) the probability of III*b* increases from 0.4 to 1.0 a factor of 2.5. Therefore, if III*a* is right, 4-methyl-3-hydroxyphenyl trimethyl ammonium ion V



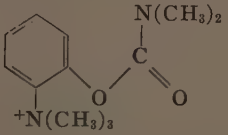
IV



V

should be only very slightly better than I, but if III*b* is correct it should be 1.25 × 2.5 × 120 = 370 times better than I, which was in fact found to be the case. Therefore III*b* is correct.

This conclusion was confirmed by measuring the binding of 4-hydroxyphenyl trimethyl ammonium ion and 2-hydroxylphenyl trimethyl ammonium ion. Assuming III*b* to be correct, we conclude that the 4-hydroxy compound cannot form the hydrogen bond and its binding constant will not be very different from I. This was found to be true: it was 1.1 times better. However, the 2-hydroxy derivative might well be considerably better than I, and it was found to be 8 times better. We therefore know that the *b* configuration of III is correct and we have an approximate position of G̤. Let us return to the dimethyl carbamate, compound II. It is clear from the approximate position of G̤, which has already been selected so that configuration II*b* must be selected if the carbonyl carbon atom is to form a covalent bond with G̤. This conclusion is strengthened by the observation that the dimethyl carbamate of the 2 hydroxy derivative (VI) is also a very good inhibitor



VI

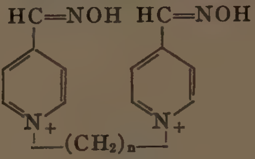
and that in this case the carbamate function contributes a binding factor of about 300.

We return now to the pyridine-aldoximes. It remains for us to show that no large intrinsic difference exists in the activity of the 2,3, and 4 derivatives. In their reaction with *p*-nitrophenyl acetate and ethyl methylphosphono fluoridate (Sarin), there is a spread of only a factor of 2 in reaction rate among the 3 compounds. The syn compound of 2-PAM (the only one sufficiently stable to study; they are readily transformed into the anti configuration) also reacts rapidly with nitrophenyl acetate but, as anticipated from the spatial relationships, is not active in reactivating inhibited acetylcholinesterase.

If we consider the reactivation of an enzyme such as chymotrypsin, where no molecular complementarity would be expected between the enzymes and the pyridine aldoxime methiodides, we should not expect these compounds to be very active, and there should be no great distinction among them. Two days at high concentration are required to reactivate chymotrypsin and, as expected, all are about equally active.

There are two aspects to the concept of increasing reactivity with a protein by introducing binding features into a molecule. If the binding does not fix the position of the functional group, activity may be promoted simply because binding increases the effective concentration. However, if the functional group is oriented, we may have either a very favorable result, as in 2-PAM and 4-PAM, or an unfavorable consequence, as in 3-PAM, depending upon whether or not the orientation is such as to fix the functional group in a position suitable for reaction.

It would appear possible to increase further the activity of 2-PAM and 4-PAM by introducing other binding features, provided we do not thereby spoil the orientation. In general, diquaternary ions are much better reversible inhibitors of acetylcholinesterase than monoquaternary ammonium ions. Poziomek *et al.*[32] utilized this principle and introduced a second quaternary center in the molecule. The bisquaternary derivatives of 4-PAM (VII)



VII

that they prepared are very much more active (in the sense of reactivating at lower concentrations) than the monoquaternary pyridine aldoximes.[32, 33]

It is our thought that the principles of molecular complementarity that we have used in this work will come to play an increasingly important role in the study of the interactions of proteins with small molecules and in the design of drugs.

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Part II. Neuromuscular and Synaptic Transmission

SOME IONIC FACTORS THAT INFLUENCE THE ACTION OF ACETYLCHOLINE AT THE MUSCLE END-PLATE MEMBRANE*

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The essence of a widely held view of neuromuscular transmission is as follows: (1) the arrival of a motor nerve action potential greatly enhances the release of acetylcholine from the motor axon terminals; (2) this chemical intermediary diffuses to the outer surface of the end-plate membrane where it combines with receptors located there; and (3) as a consequence, the end-plate membrane develops an increased ionic permeability resulting, in this case, in a diminution of the potential difference ordinarily appearing across it.

The reduction in potential difference (end-plate potential) requires the removal of a proportionate amount of the electric charge stored in the end-plate membrane capacitance. This discharge, which involves displacement of ions, can be produced by a net influx of positive charge, a net efflux of negative charge, or both. When one attempts to explain the basic mechanism involved in this transmembrane ionic displacement, two questions arise: (1) What is the identity of the moving ions? (2) At what rates do the various ionic species move across the activated end-plate membrane and how do these rates vary with time?

A few years ago, Fatt and Katz (1951) proposed that the application of acetylcholine transforms the end-plate membrane into a structure that is permeable to all ions. This special patch of activated membrane is electrically coupled to the surrounding nonend-plate membrane; hence it will act as a short circuit for the latter, thereby leading to the generation of propagated muscle action potentials. In this theory the difference in potential across the activated end-plate membrane will approach the value representing the liquid junction potential between intracellular and extracellular fluids. More recently, del Castillo and Katz (1956) have reviewed the lines of evidence supporting the "short circuit" hypothesis.

Sodium ions represent a likely source of rapidly penetrating positive charge required to generate the end-plate potential because their chemical gradient and the electric field in the inactive membrane favor inward movement. That additional ion species are involved was indicated by the early work of Fatt (1950) in which it was shown that in the absence of extracellular sodium ions, the acetylcholine-activated membrane becomes depolarized, although to a more limited extent.

With the idea of removing certain difficulties inherent in the work of Fatt (1950), I repeated his experiments in sodium-free Ringer's solution, but used intracellular recording and a high-speed microelectrophoretic method for applying acetylcholine to the outer surface of the end-plate membrane (FIGURE 1). It was found that application of acetylcholine to the end plate produced little or no depolarization when all sodium ions (and associated chloride ions) were removed from the extracellular fluid (see FIGURE 2 and Nastuk, 1953, 1955). From these results one might draw the following conclusions. (1) Depolarization of the acetylcholine-activated end-plate membrane depends almost completely on sodium ion influx. (2) The acetylcholine-produced permeability increase allows easier transmembrane movement of Na^+, K^+, and Cl^-. Hence, if Na is absent from the extracellular fluid and if $E_R = E_K = E_{Cl}$, no change in resting membrane potential (E_R) will occur since, according to A. L. Hodgkin and P. Horowicz (unpublished data)

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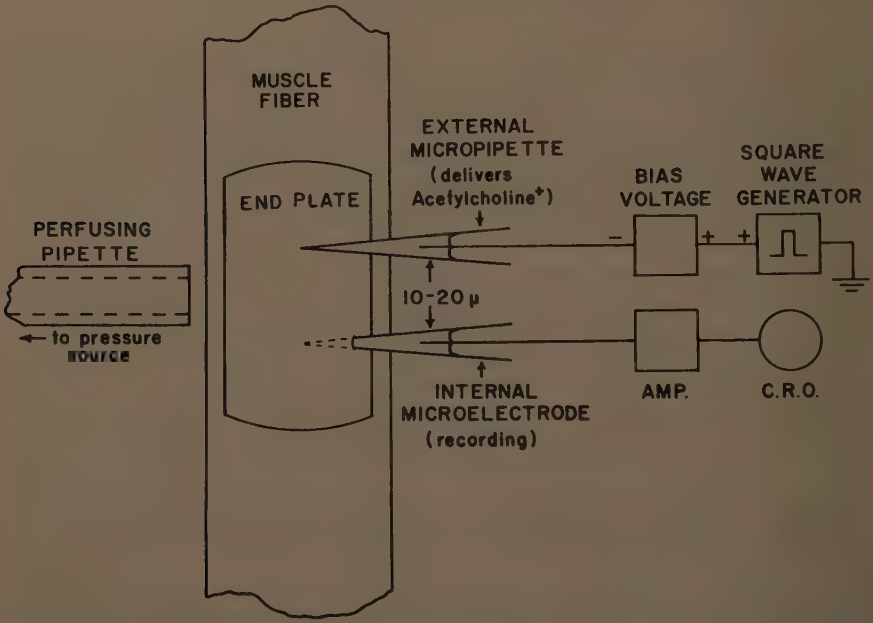

FIGURE 1. Schematic diagram of the experimental arrangement by which were recorded the changes in transmembrane potential produced by the microelectrophoretic application of acetylcholine ions to the outer surface of the end plate. A change in the composition of the extracellular fluid is produced by perfusing the muscle fiber with various solutions, which are delivered by the pipette at the left. The bias voltage prevents the diffusion of acetylcholine from the upper micropipette at the right during stand-by periods.

$$\frac{K_i}{K_o} = \frac{Cl_o}{Cl_i} = \exp.\frac{E_R F}{RT}$$

where K and Cl refer to activities of these ions inside (i) and outside (o) the cell. (3) In media of low ionic strength, such as are produced when sodium chloride is replaced by an osmotically equivalent amount of sucrose (TABLE 1), end-plate membrane receptors become insensitive. In this state they can no longer be activated by physiological amounts of acetylcholine.

At the time these results were obtained, no further evidence was available on which to base a choice of the above alternatives. Shortly thereafter, however, del Castillo and Katz (1955) showed that if muscle fibers are soaked in isotonic potassium sulfate solution, end-plate membrane receptors nonetheless still re-

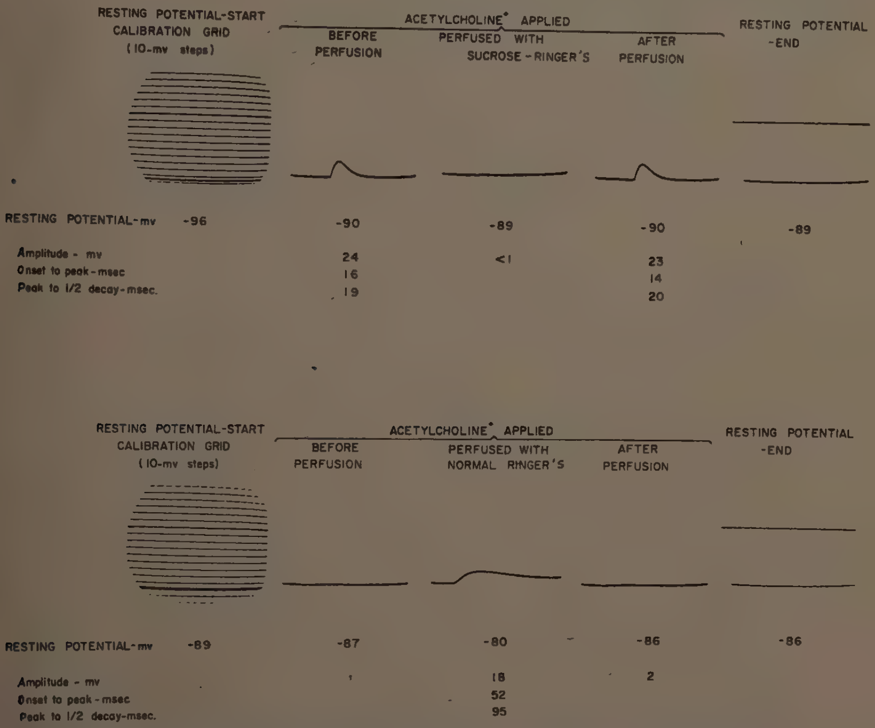

FIGURE 2. Typical responses to the application of acetylcholine$^+$ obtained at the end plate of a single muscle fiber: *above,* immersed in normal Ringer's solution and perfused with sucrose Ringer's solution; *below,* immersed in sucrose Ringer's solution and perfused with normal Ringer's solution. Note the positive relation between the depolarizing action of acetylcholine on the end-plate membrane and the presence of NaCl in the extracellular fluid. The resting potentials measured during perfusion were not corrected for the liquid junction potential formed at the periphery of the perfusion pool and the bath solution.

TABLE 1
COMPOSITION OF SOLUTIONS
(All Concentrations as mM/l.)

	Sodium Ringer's	Sucrose Ringer's
Na^+	116.55	—
K^+	2.5	2.5
Ca^{++}	1.8	1.8
Cl^-	117.1	3.6
$H_2PO_4^-$	0.45	0.20
$HPO_4^{=}$	2.55	1.15
Sucrose	—	233.4

act with acetylcholine, and produce thereby an increase in membrane permeability. These investigators were not able to specify whether the permeability increase was one restricted to potassium ions alone, or one of a general type such that all ionic species would be permitted to move easily across the end-plate membrane. However, it seems unlikely that, in their experiments, sulfate ions crossed the end-plate membrane at the same speed as potassium ions because such behavior would have resulted in no net transfer of electric charge.

TABLE 2

EFFECT OF SODIUM RINGER'S SOLUTION CONTAINING ACETYLCHOLINE (100 MG./L.) ON THE MEMBRANE POTENTIAL OF MUSCLE FIBERS IMMERSED IN SUCROSE RINGER'S SOLUTION

Membrane potential (mV)	Membrane potential after perfusion with sodium Ringer's containing acetylcholine (mV)	Zone tested
−81	−10	End plate
−77	−12	End plate
−84	−37	End plate
−84	−32	End plate
−84	−40	End plate
−96	−63	End plate
−92	−28	End plate
−34	−45	End plate
−75	−29	End plate
−85	−33	End plate
−87	−21	End plate
Av. −80	−32	
−83	−90	Nonend plate
−88	−95	Nonend plate
−84	−92	Nonend plate
−28	−54	Nonend plate
−78	−89	Nonend plate
−90	−97	Nonend plate
−94	−96	Nonend plate
−82	−86	Nonend plate
−85	−96	Nonend plate
−87	−81	Nonend plate
−85	−99	Nonend plate
Av. −80	−89	

With the aim of clarifying certain questions raised by the work described above, I have conducted new experiments using frog sartorius muscle that had been soaked in sodium-free sucrose Ringer's solution. The initial step was to determine whether end-plate membrane receptors that have been placed in a medium of low ionic strength become so insensitive to acetylcholine that their activation by this agent becomes severely limited or impossible.

After soaking the muscle for 20 min. or more in sucrose Ringer's solution, the transmembrane potential was measured at the end plates before and during their perfusion with sodium Ringer's solution containing acetylcholine at 100 mg./l. With the onset of perfusion, the transmembrane potential fell quickly to a low value, often near zero, and it reached this level in 1 to 3 sec. (TABLE 2). A strong contracture was also produced; although this event complicated the final phase of the membrane potential records, it did not invalidate the general conclusion. The conclusion reached was that if media of low ionic strength cause receptor desensitization, the process is at least rapidly reversible.

By the next set of experiments it was possible to show that in a medium of low ionic strength (sucrose Ringer's solution), receptors can be activated, and in time inactivated, by acetylcholine. The sartorius muscle, after being washed in sucrose Ringer's solution for 20 to 30 min., was immersed in sucrose Ringer's solution containing acetylcholine 100 mg./l. Three minutes later and continuing thereafter, the transmembrane potential of a series of end plates was measured before, during, and after perfusion with sodium Ringer's solution containing acetylcholine 100 mg./l. The results (TABLE 3) showed that during the early phase of exposure to acetylcholine the end-plate receptors were in the activated state because an appreciable drop in membrane potential was

TABLE 3

THE INACTIVATION OF END-PLATE MEMBRANE RECEPTORS OF MUSCLE FIBERS IMMERSED IN SUCROSE RINGER'S SOLUTION TO WHICH ACETYLCHOLINE IS ADDED (100 MG./L.)

Experiment	Elapsed time after addition of acetylcholine (min.)	Membrane potential (mV)	Membrane potential after perfusion with sodium Ringer's solution and acetylcholine, 100 mg./l. (mV)	Zone tested
1	3	−84	−79	End plate
	5	−81	−91	End plate
	7	−85	−99	End plate
2	2	−87	−57	End plate
	4	−78	−66	End plate
	13	−77	−80	End plate
	18	−79	−88	End plate
	21	−81	−92	End plate
	23	−77	−76	End plate
3	1	−73	−42	End plate
	4	−75	−55	End plate
	6	−84	−55	End plate
	10	−91	−72	End plate
	13	−92	−99	End plate
	16	−94	−71	End plate
	23	−80	−69	End plate
	29	−80	−73	End plate
	34	−86	−87	End plate
	35	−77	−69	End plate
	45	−89	−92	End plate
1, 2, 3	9	−87	−93	Nonend plate
	11	−84	−92	Nonend plate
Controls	5–10	−88	−98	Nonend plate
	5–10	−90	−88	Nonend plate
	5–10	−86	−94	Nonend plate
	25	−81	−80	Nonend plate
	26	−81	−87	Nonend plate
	42	−92	−103	Nonend plate

All muscles were immersed in sucrose Ringer's solution for 35 to 130 min. before the addition of acetylcholine.

produced when the extracellular sodium ion concentration was increased via the perfusion. However, after longer exposure to acetylcholine, newly selected end plates, when similarly tested, no longer gave signs of appreciable depolarization, indicating that receptor inactivation had occurred. At this stage the bath was drained and the muscle was washed with sucrose Ringer's solution containing no acetylcholine. Early in the washout period, the perfusion of end plates with sodium Ringer's solution containing acetylcholine (100 mg./l.) gave rise to a marked depolarization (TABLE 4). From this it was concluded that the acetylcholine-produced receptor inactivation can be rapidly reversed if acetylcholine is removed from the extracellular fluid.

At this point one may reconsider the results of the experiments previously described and illustrated in FIGURE 2, wherein acetylcholine was applied electrophoretically to end plates of fibers soaked in sucrose Ringer's solution. On the basis of present experiments it now seems reasonable to take the position that in these early experiments the receptors might have been activated, that thereby an increase in membrane permeability was produced, and that the increase was selective for potassium ions or for both potassium and chloride ions. If, as seems to be the case, the distribution of these ions between intracellular and extracellular phases is such that their equilibrium potentials lie at or close to the resting potential of the end-plate membrane, then an increase in membrane permeability to either or both of these ions would cause little or no change in membrane potential, although a decrease in membrane impedance would occur.

TABLE 4

THE REACTIVATION OF END-PLATE MEMBRANE RECEPTORS OF MUSCLE FIBERS AFTER THE REMOVAL OF ACETYLCHOLINE*

Experiment	Elapsed time after removal of acetylcholine (min.)	Membrane potential (mV)	Membrane potential after perfusion with sodium Ringer's containing acetylcholine (mV)	Zone tested
1	20	−90	0†	End plate
	20	−88	0†	End plate
2	2	−76	−79	End plate
	6	−81	−31	End plate
	8	−76	−22	End plate
	10	−75	−29	End plate
3	5	−94	−18†	End plate
	10	−85	−33	End plate

* All muscles had been immersed in sucrose Ringer's solution containing acetylcholine (100 mg./l.) for 11 to 45 min.

† Microelectrode pulled out of the fiber.

Attractive though this explanation may be, it leaves unexplained one puzzling complication. As mentioned earlier, when all sodium ions (with associated chloride ions) are removed from the extracellular fluid, microelectrophoretic application of acetylcholine sometimes caused a small reduction of membrane potential at the end plate (FIGURE 2). Del Castillo and Katz (1955), who obtained similar results, speculated that this residual depolarization might be the result of incomplete removal of the extracellular sodium ions. It appears that this explanation is no longer satisfactory, judging from results of new experiments described below.

The technique for removing sodium ions from the extracellular fluid involves soaking the sartorius muscle in frequently changed baths of sucrose Ringer's solution. In earlier work, membrane potential measurements were usually begun after 30 min. of soaking in this medium; occasionally, low resting potentials were observed, sometimes at a level of -10 to -20 mV. At the time it was assumed that this unexpected result was a sign of some unsuspected fault in the preparation, an assumption I now believe to be unwarranted. During the present experiments it has consistently been found that fibers showing low resting potentials during the early phase of sodium ion washout slowly recover their normal resting potentials until, after 2 hours or more in sucrose Ringer's solution, values in the -85 to -100 mV range are obtained. A few observations indicated that this recovery of the normal resting potential proceeds more rapidly for fibers located near the muscle edge.

Apparently muscle fibers are not damaged by soaking for 2 to 4 hours in sucrose Ringer's solution because, on reimmersion in normal sodium Ringer's solution, excitability and ability to conduct action potentials are quickly restored. Resting potentials of long-soaked fibers measured shortly after return to normal Ringer's solution were found to exceed 100 mV (FIGURE 3) and, with time, this hyperpolarization diminishes. If, at the completion of the drift in membrane potential, the muscle was once again returned to the sucrose Ringer's solution, it was found that the resting potentials were again appreciably reduced.

The interpretation of the preceding results would be difficult were it not for the recent work of Hodgkin and Horowicz (unpublished). I am greatly indebted to these investigators for granting me an opportunity to review their manuscript and to refer to certain portions of it.

There is connection with results reported here and the findings of Hodgkin and Horowicz, who showed, using single frog muscle fibers, that the value of the resting potential is governed by extracellular-intracellular concentration ratios of potassium and chloride ions. Consider on this basis the effect of immersing a muscle fiber in sucrose Ringer's solution. The potassium equilibrium potential E_K as given by $E_K = \frac{RT}{F} \ln \left(\frac{K_o}{K_i}\right)$ would amount to -100 mV if one takes $K_o = 2.5$ mM/l. and $K_i = 140$ mM/l. The sucrose Ringer's solution contains 3.6 mM/l. of chloride added in the form of calcium chloride. Hence, the chloride equilibrium potential E_{Cl} as given by $E_{Cl} = \frac{RT}{F} \ln \left(\frac{Cl_o}{Cl_i}\right)$ would amount to -15 mV if one takes $(Cl_i) = 2$ mM/l. Using the work of Hodgkin and Horowicz as a basis, one would expect the membrane resting potential, at the start of the immersion period, to lie between -100 mV and -15 mV and the value would be closer to the latter figure. With time, a Donnan equilibrium would be reestablished wherein $(K_o)(Cl_o) = (K_i)(Cl_i)$. This condition is achieved by outward migration of KCl, which efflux will continue until Cl_i is approximately 0.06 mM/l., $K_i = 138$ mM/l., and the resting potential is raised once more to such a degree that it lies near the value for

E_K. If the soaked-out muscle is now returned to normal Ringer's solution, the chloride equilibrium potential, E_{Cl}, will be greater than either the resting or potassium equilibrium potentials, and thus the membrane potential rises (FIGURE 3).

Of particular interest is the effect of applying acetylcholine to the end-plate membrane at various times following immersion in sucrose Ringer's solution. Results of such tests, given in TABLE 5, are in agreement with earlier observations of del Castillo and Katz (1955), who showed that application of acetylcholine, under certain conditions, can cause an increase of transmembrane potential at the end plate. For the fully equilibrated fiber, it can be seen that application of acetylcholine at 100 mg./l. caused a reduction in transmembrane potential. The fact that similar results were obtained using 20 mg./l. acetylcholine make it unlikely that this change is due to penetration of the quaternary ammonium ion itself, or its hydrolytic products. Application of acetylcholine to nonend-plate sites gave a negative result regardless of the value of the membrane potential.

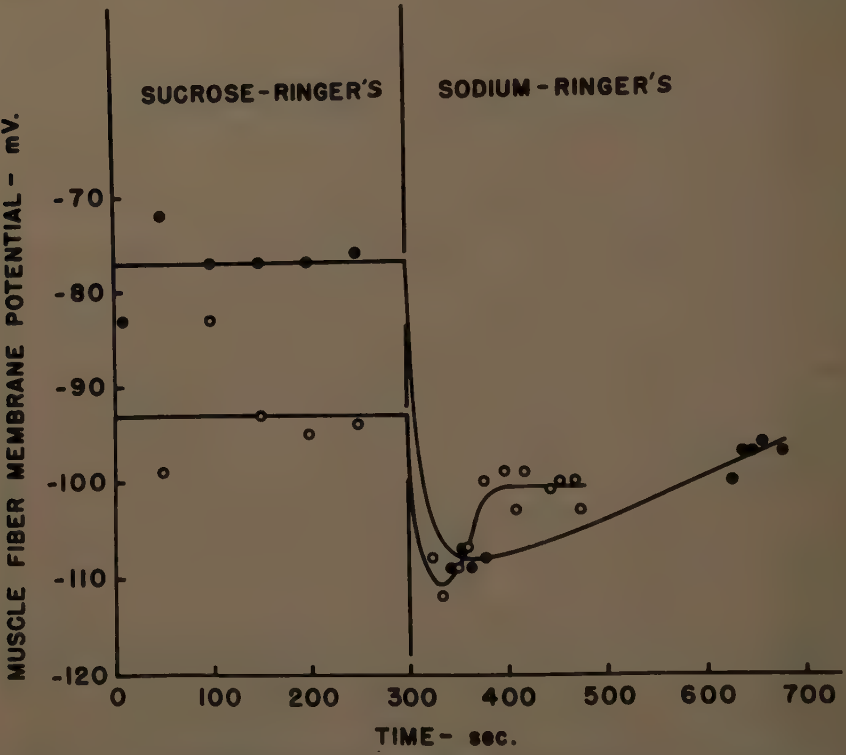

FIGURE 3. Two experiments showing the increase in transmembrane potential produced when a frog sartorius muscle equilibrated in sucrose Ringer's solution was placed in normal Ringer's solution. The solid circles represent transmembrane potentials measured in individual fibers sampled from the muscle during the course of one experiment; the open circles represent the results from another experiment of the same type but using another muscle.

The increase in transmembrane potential caused by application of acetylcholine to the "chloride-depolarized" end-plate membrane could be interpreted as resulting from an increase in end-plate membrane permeability to both potassium and chloride ions although, under these conditions, the mobility of potassium ions would exceed that for chloride. The above explanation is not tenable for the case of the fiber fully equilibrated in sucrose Ringer's solution where the transmembrane potential assumes a value near that of the potassium or chloride equilibrium potentials. It may therefore be postulated that the acetylcholine-activated membrane becomes permeable to at least one more ion in addition to potassium and chloride. One would suspect, on the basis of recently published work of Frankenhaeuser and Hodgkin (1957), of Hodgkin and Keynes (1957), and of Fatt and Ginsborg (1958), that Ca^{++} is the ion in question.

One may again recall that, in earlier work in which acetylcholine was applied electrophoretically to end plates of muscle fibers soaked in sucrose Ringer's solution (FIGURE 2), a small depolarization was produced on some occasions. It now seems that this result represents the uncertain sign of an event better demonstrated by the microperfusion method. The smallness and uncertainty of the depolarization produced by the microelectrophoretic method might be explained on two bases: (1) the time course of penetration of the unknown ion is too slow to be important in membrane activations lasting a few milliseconds; (2) receptor activation must be widespread and sustained in order to allow a substantial quantity of the unknown ion to move across the membrane.

TABLE 5
EFFECT OF ACETYLCHOLINE PERFUSION (100 MG./L.) ON THE MEMBRANE POTENTIAL OF MUSCLE FIBERS IMMERSED IN SUCROSE RINGER'S SOLUTION

Zone tested	No. of fibers	Average membrane potential (mV)		
		Before perfusion	During perfusion max. effect	After sustained perfusion
End plate	16	−90	−78	−85
End plate	5	−80	−71	−68
End plate	3	−70	−72	−70
End plate	2	−62	−72	−63
End plate	1	−30	−66	−50
End plate	2	−19	−77	−49
Nonend plate	10	−80	−79	−77

The final set of experiments was designed to explore further the proposal originally made by Fatt and Katz (1951), who visualized the acetylcholine-activated end-plate membrane as "broken down" in the sense that it allowed easy passage through it of all ion species.

One might imagine a broken-down membrane to be one in which there occurs in reality a transient disarray of the lipid and protein molecules of which it is composed. It seems more reasonable to suppose that the permeability change involves the widening of transverse channels in the membrane through which ions pass, perhaps with a mobility approaching the value characteristic of free solution.

In an attempt to obtain more information concerning the nature of the permeability change, I have used a series of univalent cations to test the relation between ionic size and ability to penetrate the acetylcholine-activated end-plate membrane. One procedure involved washing the muscle in sucrose Ringer's solution and then measuring the transmembrane potentials at end-plate and nonend-plate sites during perfusion with modified Ringer's solution that contained acetylcholine (100 mg./l.), and in which the sodium chloride was replaced with equivalent amounts of various substitute salts whose cations varied in size. A decrease in transmembrane potential of the end plate produced during the perfusion was taken as evidence that the cation substituting for sodium had penetrated the end-plate membrane. In other experiments, the muscle was kept in a bath of sodium Ringer's solution, and fibers were perfused both on and off the end plate with the substituted Ringer's solution containing acetylcholine (100 mg./l.). Failure to cause depolarization of the end plate was taken as indication that the cation that substituted for sodium in the perfusion solution acted as a competitive inhibitor of acetylcholine at the receptor sites. In one additional experiment utilizing a muscle immersed in sodium Ringer's solution, end-plate and nonend-plate sites were perfused with ammonium chloride Ringer's solution in order to test the power of the ammonium ion as a receptor activator or blocker.

There are complications in experiments of the above type which, in the present work, have been only partially overcome. First, potentials of appreciable magnitude may appear at the tip of a microelectrode making contact with sucrose Ringer's solution. This effect was minimized by use of freshly prepared microelectrodes having tips of the maximum permissible diameter. Second, a liquid junction potential was created during perfusion at the point where the pool of perfusate joined the sucrose Ringer's solution. This potential was eliminated by measuring the transmembrane potential between a point just inside the membrane and another point just outside it in the pool of perfusate. Third, the increase in extracellular chloride (or bromide or iodide) ion during perfusion caused an increase in transmembrane potential. Fourth, application of acetylcholine to end plates of muscle fibers long immersed in sucrose Ringer's solution can cause a small diminution in transmembrane potential (TABLE 5). Fifth, during a perfusion lasting several seconds, a small depolarization might be produced by influx of a slow-moving ion. The same ion might not make an appreciable contribution to the total ionic charge displaced in a depolarization process lasting 1 to 2 msec. (as during neuromuscular transmission). Hence the upper size limit for ions participating in neuromuscular transmission is likely to be below that indicated by perfusion experiments. Sixth, some cations are competitive inhibitors of acetylcholine at receptor sites.

Considering the preceding, it seems advisable at present to report the results of the perfusion experiments in qualitative form. In terms of ability to depolarize the acetylcholine-activated end-plate membrane, the order is: ammonium = strong; tetramethylammonium = moderate; trimethylethylammonium, trimethylethanolammonium (choline), dimethyldiethanolammonium = weak; and diethyldiethanolammonium, trimethylphenylammonium = weak to nil. The penetration of tetraethylammonium and tetrapropylammonium could not be ascertained, because both these ions completely inhibited receptor activation by acetylcholine.

By means of other experiments done in the absence of acetylcholine, it was shown that trimethylphenylammonium and trimethylethanolammonium (choline) cause receptor activation followed by inactivation. Ammonium and diethyldiethanolammonium did not cause measurable activation or block of end-plate membrane receptors. Trimethylethylammonium and dimethyldiethanolammonium were not tested under these conditions.

At this point it is important to add that others who have been investigating certain fundamental aspects of the neuromuscular transmission process have found that the sodium ions in the extracellular fluid can in some respects be replaced by ammonium (Furukawa *et al.*, 1957) or by hydrazine (Koketsu and Nishi, 1958).

Surveying the results of the sodium substitution experiments and keeping in mind the limitations mentioned above, one conclusion may be drawn: that quaternary ammonium ions, whose diameter is not very much greater than that of tetramethylammonium, do not penetrate the activated end-plate membrane readily. Such behavior would seem to militate against the idea that the end-plate membrane undergoes temporary dissolution during acetylcholine activation. One may be encouraged to believe that systematic study of the physicochemical characteristics of ions that can substitute for those normally appearing in the extracellular and intracellular fluids will provide basic information of great value in the understanding of ion transport mechanisms and their regulation.

Acknowledgments

I thank Raphael Lorente de Nó of the Rockefeller Institute, New York, N. Y., for his kindness in providing me with some of the amines. I also express my gratitude to J. T. Alexander of this department for his unfailing technical assistance.

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THE PRESYNAPTIC ELEMENT IN NEUROMUSCULAR TRANSMISSION*

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During the past two decades the neuromuscular blocking drugs have proved to be an exceedingly popular subject for pharmacological and physiological study. Oddly, the opposite aspect, that is, the facilitation of neuromuscular transmission by drugs, has been given relatively little attention. This topic was first exposed to serious scrutiny when Brown *et al.* (1936) demonstrated the effect of physostigmine in enhancing the contractile response of mammalian striated muscle. Immediately thereafter Brown (1937) ascertained that the mechanism of this effect results from the conversion of each single muscle response to a repetitive one. Just two years earlier the clinical expression of neuromuscular facilitation was recorded when Walker (1934) described the dramatic alleviation of the myasthenic syndrome through the administration of either physostigmine or its newly synthesized congener neostigmine. Later research in man (Harvey and Lilienthal, 1941) related these laboratory and clinical observations, and it was revealed that the transmission deficit in myasthenia gravis could be temporarily repaired by neostigmine. At this time Eccles and his co-workers (1941) employed physostigmine to examine the newly discovered end-plate potential and found that the alkaloid greatly prolonged junctional negativity. This seemed a reasonable consequence of the drug's anticholinesterase (anti-ChE) activity, and Eccles and MacFarlane thereafter (1949) showed a similar effect for other carbamates and di-isopropylfluorophosphate (DFP). On the basis of this work, the prolongation of junctional negativity is often considered synonymous with ChE inhibition. Whether or not this evidence assures a causal relationship may be questioned. In any case, the findings of Eccles *et al.* (1941) are important, for they disclose a mechanism by which physostigmine and similar drugs cause the junctional region to initiate repetitive discharges in response to individual nerve volleys.

In 1946 W. C. Wescoe and I were engaged in an evaluation of DFP in the management of myasthenia gravis. Our efforts only impressed us the more with the remarkable therapeutic efficacy of neostigmine in this disease. Consideration of structure-activity relationship led to a search of the existing literature that revealed, for neostigmine and its congeners, only a concern with the dependence of ChE inhibition on the presence of an alkyl carbamino group; strangely, the possible functional importance of the quaternary ammonium grouping was unmentioned. Simple experiment showed (Riker and Wescoe, 1946) the functional nature of the quaternary ammonium grouping. It was found that chronically denervated cat striated muscle is susceptible to direct excitation by neostigmine, just as it is by acetylcholine and other quaternary ammonium ions in which the N atom is substituted with 3 methyl groups. To

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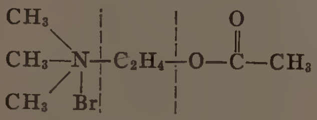
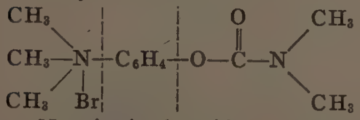
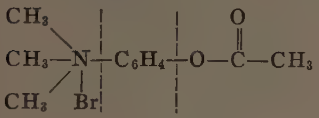
assess the importance of this direct effect of neostigmine we were curious to examine a neostigmine-type molecule lacking the carbamino groups. For this purpose John Aeschlimann of Hoffmann-La Roche, Inc., Nutley, N. J., supplied us with the 3-acetoxy analogue and later with its unesterified phenolic derivative, 3-hydroxy phenyltrimethylammonium ion (3-OH PTMA); these structural interrelationships are shown in FIGURE 1. In simultaneous studies, Riker and Wescoe (1950) and Randall and Lehmann (1950) and Randall (1950) made it apparent that both of these substances exhibit the characteristic actions of neostigmine at the mammalian neuromuscular junction. These effects, as a function of dose, include the following: striking potentiation of the maximal twitch response, an efficient anticurare action, the initiation of spontaneous fasciculations, the blockade of neuromuscular transmission, and the excitation of chronically denervated muscle.



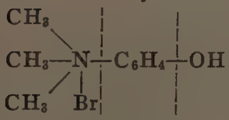
Acetylcholine bromide



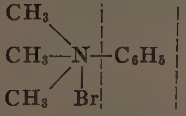
Neostigmine bromide



3-Acetoxy PTMA



3-Hydroxy PTMA



PTMA

FIGURE 1

Voluminous pharmacological evidence dating back to 1869 (Crum-Brown and Fraser) relates the latter two effects to the action of the methonium ion, Me_3N^+. Therefore, for compounds containing the methonium grouping, evaluation of neuromuscular facilitation demands a dissociation of the complicating blocking action common to methonium ions. For 3-OH PTMA the blocking action occurs in the same dose range in which facilitation appears; consequently, the latter may be diminished or obscured by the former, depending on the magnitude of the dose.

Methods

The basic method employed in the experiments to be reported was the cat gastrocnemius preparation described by Riker *et al.* (1957, 1959). The muscle remained *in situ* with the leg circulation restricted to the gastrocnemius; all injections were made intra-arterially close to the muscle. Continued anesthesia and persistent anesthetic effects were avoided by preliminary cord section at L4 under cyclopropane anesthesia. In most experiments the nerve to the gastrocnemius muscle was stimulated *in toto* or in part. In some experiments activation of motor elements was achieved via stimulation of fibers of ventral root L7. When exposure of the ventral root was necessary, either one of two preparatory procedures was followed. In one, preliminary decerebration was performed under cyclopropane anesthesia, after which anesthesia was discontinued and the preparation completed; in the other, chloralose anesthesia was used. Results obtained with either method were comparable.

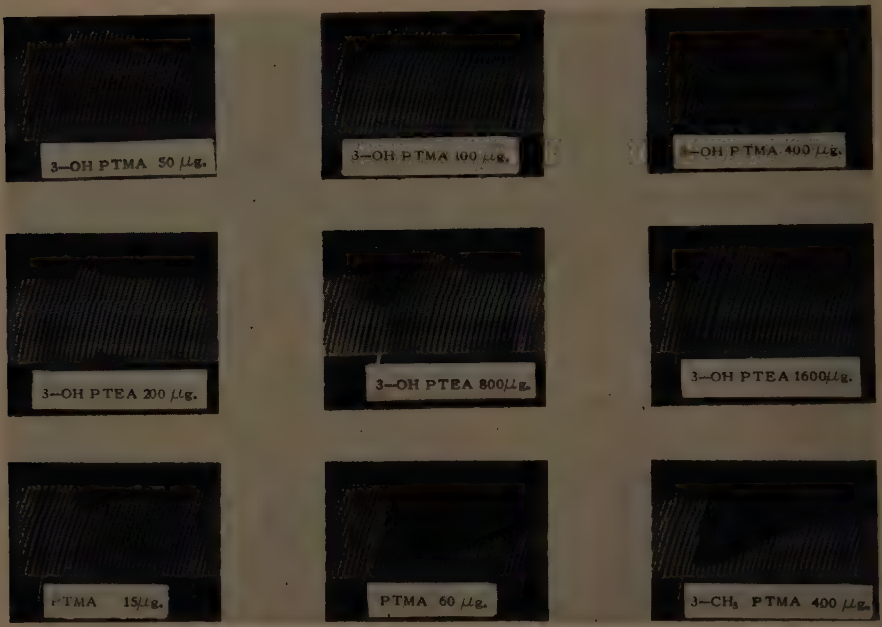


FIGURE 2. Quaternary ammonium ion-evoked potentiation and/or depression of the isometric contraction of gastrocnemius. The results obtained in 3 experiments are depicted. In each case, single maximal nerve shocks were delivered to the sciatic nerve every 7 sec. as square waves of 1-msec. duration. The downward deflection signals injection (Riker *et al.*, 1957). Reproduced by permission from *The Journal of Pharmacology and Experimental Therapeutics.*

Structural Correlates for the Blocking and Potentiating Effects of Certain Quaternary Ammonium Ions on Neuromuscular Transmission

FIGURE 2 shows the isometric twitch response of cat gastrocnemius muscle stimulated indirectly by single maximal shocks. In the first two cuts of the third row may be seen the characteristic methonium ion action, illustrated by the blocking effect of PTMA. Considerable evidence exists (Riker, 1953) to show that the blocking action of methonium ions is associated with an initial excessive depolarization of the postsynaptic membrane. This intense direct blocking action of PTMA contributes significantly to the more complex action of the related phenolic quaternary ammonium ion, 3-OH PTMA. Accordingly, the striking twitch potentiation caused by 3-OH PTMA may be seen in FIGURE 2, row A. However, it is to be noted here that the blocking action of this methonium ion is discernible even when the net effect is one of potentiation. Increasing the dose of 3-OH PTMA permits the blocking action to appear more prominently, and diphasicity of response is obvious. Most important is the emergence of the simple fact that the contribution of the OH group is unique; this grouping endows this neuromuscular blocking molecule with a new property, namely, the capacity to facilitate transmission.

These findings, in accord with those of Nastuk and Alexander (1954) based on experiments with Tensilon* chloride lead to the conclusion that the facilitation of neuromuscular transmission is independent from any direct depolarizing effect. Certainly, if depolarization and facilitation were related, it is reasonable to expect that some dose of PTMA would readily display this effect; such is not the case. The separability of depolarizing potency and facilitation may be tested in another way. Utilizing the principle long ago employed by Burn and Dale (1915), the depolarizing potency of 3-OH PTMA can be dissipated by exchanging the methyl groups for ethyl groups, forming 3-hydroxyphenyltriethylammonium ion (3-OH PTEA). When 3-OH PTEA is tested on the cat gastrocnemius preparation, the response to indirect single nerve volleys is one of pure potentiation (Riker *et al.*, 1957). This is shown in row B of FIGURE 2. This uniphasic response occurs over the entire effective dose range of 3-OH PTEA and stands in sharp contrast to the biphasic effects of 3-OH PTMA. The distinctive contribution of the OH group is emphasized once again by the fact that the unsubstituted phenyltriethylammonium ion (PTEA) is without either facilitatory or depressant effects on neuromuscular transmission.

From a chemical viewpoint, it may be thought that the OH group is not specifically reactive, but serves merely to achieve some critical onium charge density necessary for facilitation. Although the failure of PTMA to effect facilitation in any dose makes this unlikely, the specificity of the OH group may be tested by preparation of the isostere, 3-methylphenyltrimethylammonium (3-CH_3PTMA). This ion, like PTMA, when tested over a range of doses, causes only transmission block; an example of this may be seen in FIGURE 2 in the last tracing of the third row.

Quantitative comparison of the blocking potencies of PTMA, 3-OH PTMA, and 3-CH_3PTMA shows that the blocking potency of the *meta* derivative is only 17 per cent of unsubstituted PTMA (FIGURE 3). From this it may be inferred that these substitutions, in accord with chemical theory, have attenuated the onium charge and have thus weakened the depolarizing activity. However, the point remains that all possess a common blocking action, as indicated by the parallelism of their dose-response regression lines. It must be concluded that the neuromuscular facilitation evoked by the phenolic quaternary ammonium ions is distinct from whatever depolarizing property these ions may exert.

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* Registered trademark of Hoffmann-La Roche, Inc., Nutley, N. J., for a preparation of (3-hydroxyphenyl)dimethylethylammonium chloride, which is a specific anticurare agent.

The Postsynaptic Depolarizing Potencies of Certain Quaternary Ammonium Ions

Data showing the equivalent depolarizing potencies of 3-OH PTMA and 3-CH_3PTMA have been obtained in another way and agree remarkably well with the differences depicted in FIGURE 3 (Riker *et al.*, 1957). The fibrillary discharges of chronically denervated cat muscle have been used to detect and assay depolarizing activity. The spontaneous fibrillary discharge of such a fiber can be surprisingly regular and stable over long periods of time. On exposure to depolarizing substances such as acetylcholine, decamethonium (C10), or PTMA, the frequency is sharply but briefly increased, depending on the dose. If the dose is sufficiently large, a maximal discharge abruptly ceases, and quiescence prevails. This entire effect is illustrated for PTMA and C10 in FIGURE 4. From this the similar depolarizing effect of these ions is apparent. Similarly, assay of 3-OH PTMA and 3-CH_3PTMA with reference to PTMA (FIGURE 5) shows that the depolarizing potency of the two substituted ions is

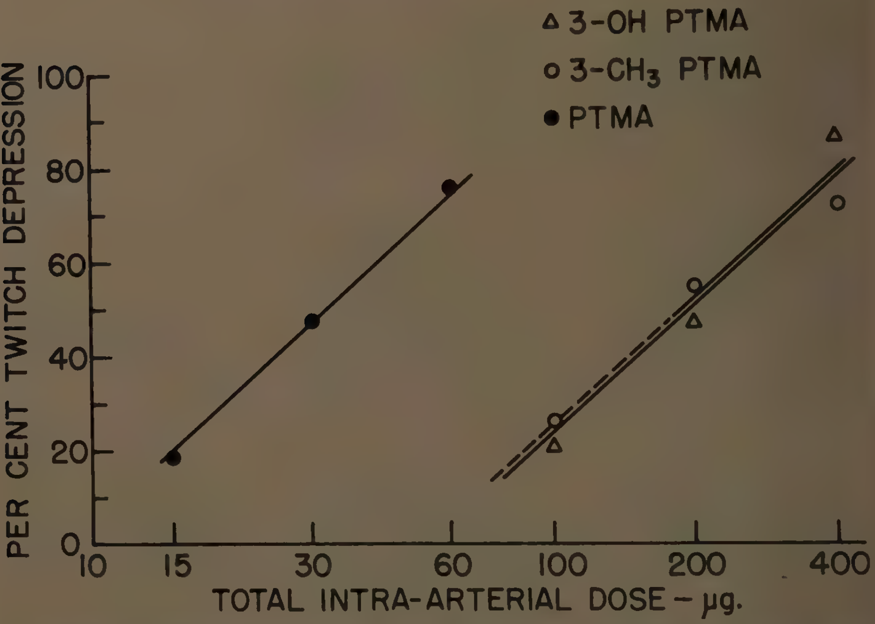

FIGURE 3. Neuromuscular blocking action of PTMA, 3-OH PTMA, and 3-CH_3PTMA. Each point represents the average of several experiments. The average number of observations for each point is 7 (range: 6 to 8). The value of the combined slopes is 0.89. The potency of 3-OH PTMA, relative to PTMA, is 17.4 per cent, with 95 per cent confidence limits at 13.5 and 22.4 per cent. The potency of 3-CH_3PTMA, relative to PTMA, is 16.5 per cent, with 95 per cent confidence limits at 13.4 and 20.4 per cent (Riker *et al.*, 1957). Reproduced by permission from *The Journal of Pharmacology and Experimental Therapeutics.*

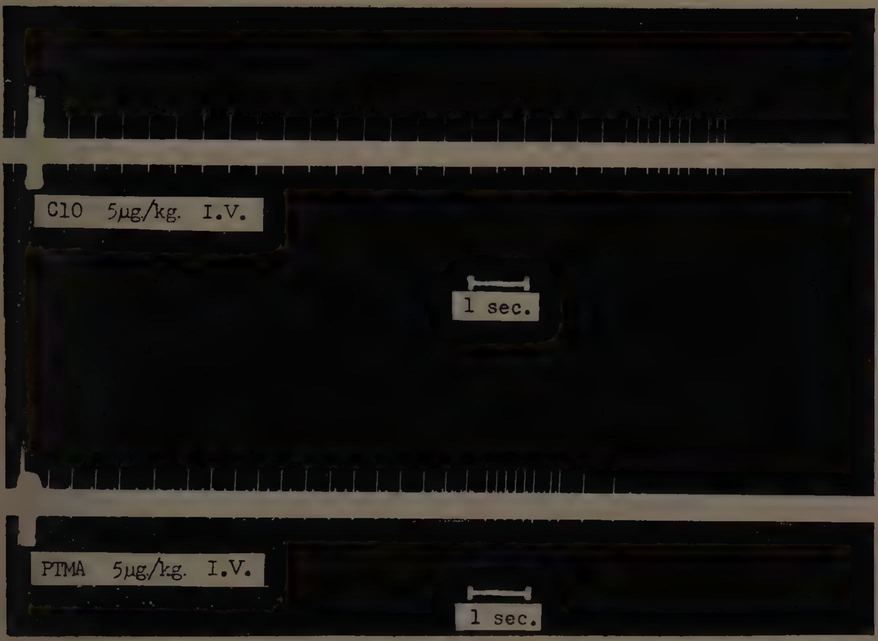

FIGURE 4. Depolarizing activity of C10 and PTMA. Recording from single fiber of tibialis denervated 12 days previously. The points of intravenous injection are marked by light streaks at the extreme left of each tracing. The same fiber was used for each observation. Note the regularity of the spontaneous fibrillary discharge. The control fibrillary discharges are not shown, since the discharges immediately after injection and prior to the onset of drug action continue exactly as in the control (Riker *et al.*, 1957). Reproduced by permission from *The Journal of Pharmacology and Experimental Therapeutics.*

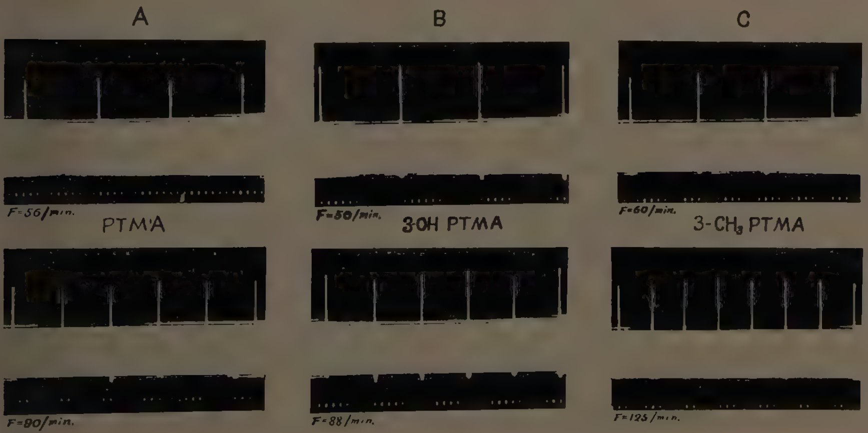

FIGURE 5. Comparison of depolarizing potencies of PTMA, 3-OH PTMA, and 3-CH_3-PTMA on denervated muscle. Recorded from single fiber of tibialis denervated 12 days previously. The control fibrillary discharge frequency, prior to each intravenous injection, is shown in the upper records. The peak effect after each injection is shown below. Time base: 1 sec. From Riker *et al.*, 1957. Reproduced by permission from *The Journal of Pharmacology and Experimental Therapeutics.*

only one-fifth that of PTMA. In addition, it has been found by this method that the depolarizing activities of 3-OH PTEA and PTEA are negligible.

The Production of Repetitive Discharge by the Phenolic Quaternary Ammonium Ions

That the phenolic quaternary ammonium ions 3-OH PTMA and Tensilon cause twitch potentiation by initiating repetitive discharge in response to single nerve volleys has been demonstrated for cat and frog muscle by Riker and Wescoe (1950) and by Nastuk and Alexander (1954). FIGURE 6 depicts the repetitive discharge that occurs within a motor unit after the injection of either PTMA, 3-CH_3PTMA, 3-OH PTMA, or 3-OH PTEA. Also shown is the relationship between the electric activity in the muscle and the contractile tension developed. In the first column it may be seen that the strong depolarizer PTMA elicits, even in a small dose, a brief asynchronous repetition that is rapidly suppressed by transmission block. Synchronous repetition and, therefore, appropriate fusion of the contractile response to increase tension cannot readily be produced with any dose of PTMA. In the second column of FIGURE 6 it may be seen that the course of action for 3-CH_3PTMA is identical to that of PTMA; the biphasic effect of 3-OH PTMA may be noted in the third column. In the second postinjection sweep the net effect, as reflected by the muscle tension, is potentiation, but transmission within the unit from which electric pickup was made is severely depressed. Throughout this particular record the electric repetition is essentially asynchronous, although not to the same degree as after PTMA. With smaller doses of 3-OH PTMA the repetitive discharge becomes more synchronous, transmission block is diminished, and potentiation improves.

In contrast with the methonium ions the effect of 3-OH PTEA, shown in the last column of FIGURE 6, is to cause an electric repetition that is highly synchronous and is associated with a sustained mechanical potentiation. There is no sign of transmission depression. Noteworthy is the synchronous repetitive response of the muscle fiber population, following 3-OH PTEA. This occurs regularly with 3-OH PTEA over a wide dosage range and is sharply different from the highly asynchronous response to PTMA.

In agreement with the simple kymograph experiments, the electric recordings indicate that methonium ion depolarization is not per se an appropriate mechanism for the facilitation of neuromuscular transmission. On the other hand, the effects of 3-OH PTEA do disclose that it is possible to facilitate neuromuscular transmission by chemical means as well as by depolarization.

The Origin of the Repetitive Discharge Produced by the Phenolic Quaternary Ammonium Ions

The fact that 3-OH PTEA can evoke a neuromuscular facilitation that is commensurate with that which must occur in physiological circumstances, that is, without depression or blockade of transmission, makes 3-OH PTEA an attractive reagent. The logical question that next arises concerns the site of 3-OH PTEA action. Since the ion is without effect on the postsynaptic membrane, as revealed by the experiments with chronically denervated muscle, and

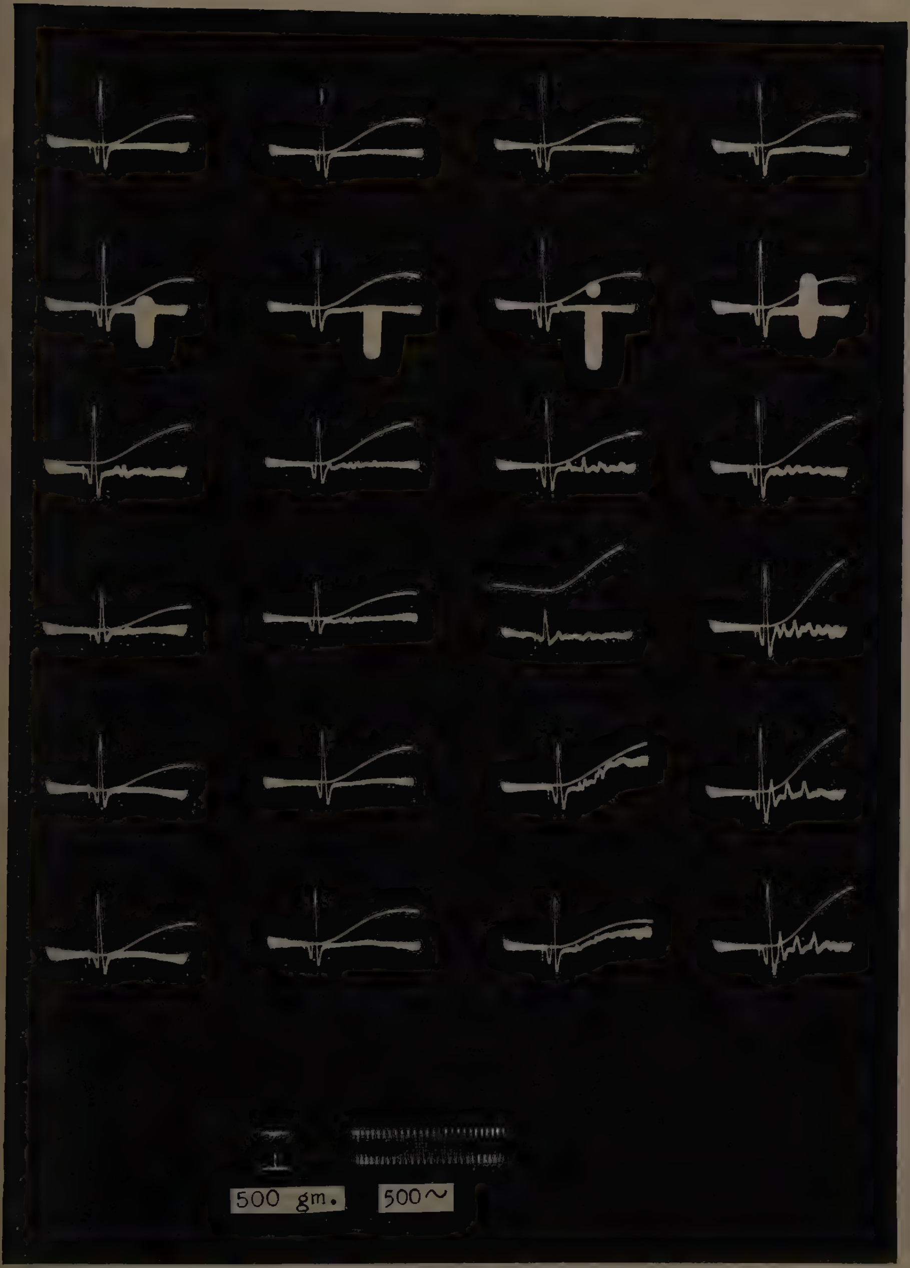

FIGURE 6. Effects of PTMA, 3-CH_3PTMA, 3-OH PTMA, and 3-OH PTEA on electric and mechanical activity of gastrocnemius. Each pair of sweeps depicts the strain gauge deflection and muscle action potential. The nerve to medial head was stimulated every 4.3 sec. with supramaximal shocks. The sequence of effects is arranged in columnar fashion from above downward. From left to right the columns are: PTMA, 3-CH_3PTMA, 3-OH PTMA, and 3-OH PTEA. The doses were: PTMA, 15 μg.; 3-CH_3PTMA, 60 μg.; 3-OH PTMA, 30 μg.; and 3-OH PTEA, 800 μg. The points of injection are marked by light streaks. Two control sweeps are shown prior to each injection. For columns 1 and 2 the four postinjection sweeps occur at 4.3, 8.6, 47.3, and 73.1 sec.; for columns 3 and 4 the four postinjection sweeps occur at 4.3, 8.6, 47.3, and 90.3 sec. From Riker *et al.*, 1957. Reproduced by permission from *The Journal of Pharmacology and Experimental Therapeutics.*

because the 3-OH PTEA-conditioned response of a population of muscle fibers is always highly synchronous, an effect on some integrative structure is presumed. Therefore, the presynaptic terminals of the motor nerve appear to be a likely focus for 3-OH PTEA action. Encouragement for this idea was derived from the earlier experiments of Masland and Wigton (1940). These investigators demonstrated that neostigmine affects the motor nerve terminal so as to cause a single volley to be followed by a train of discharges. This they detected by monitoring antidromic discharges occurring in appropriate ventral root fibers. Eccles *et al.* (1942) demonstrated a similar effect with physostigmine, but Lloyd (1942), repeating these experiments, concluded that the repetitive action on nerve after physostigmine reflected retrograde activation of nerve by muscle. The experiments by Lloyd (1942) are open to criticism since diallyl barbituric acid (Dial) was used as an anesthetic. The deleterious effect of barbiturate on repetitive excitation of the motor nerve terminal is shown by experiments described below.

To examine the behavior of the motor nerve, the experimental technique of Masland and Wigton (1940) was adapted. The experimental schema is illustrated in FIGURE 7. In some experiments the primary nerve shock was applied to L7 ventral root fibers. Stimulation of the entire popliteal nerve was made on those occasions when simultaneous recording of muscle tension was desired.

FIGURE 8 illustrates the characteristic effect of 3-OH PTEA on motor nerve. Immediately after intra-arterial injection of 3-OH PTEA, the first nerve volley evokes repetitive spikes that appear antidromically in the ventral root fibers. The peak frequency of the repetitive nerve discharge is between 150 and 200/sec. and is quickly achieved; from this the repetition frequency diminishes gradually. Repetitive activity in the muscle parallels that in the nerve. A distinctive aspect of the 3-OH PTEA effect is that repetition in nerve does not occur unless there has been a primary volley. FIGURE 9 also displays the effect of 3-OH PTEA on motor nerve, but in this case the orthodromic stimulus was applied to a fine ventral root filament and electric recording in the muscle made from the small area activated. In this circumstance, the records from the ventral root filament disclose that the repetitive antidromic spikes are precedent to each muscle potential and negate the possibility of retrograde activation of nerve by muscle. More significantly, the experiment reveals that the motor nerve terminal is the site of 3-OH PTEA action and, from the earlier work of Masland and Wigton (1940), Feng and Li (1941) and Eccles *et al.* (1942), it is reasonable to include neostigmine and physostigmine in the category of drugs that enhance neuromuscular transmission by an action on the motor nerve terminal. In turn, the mere fact that the motor nerve terminal is susceptible to these drugs strongly suggests that the terminal is a distinct functional entity.

Delineation of the Motor Nerve Terminal as a Functional Entity

The concept of the motor nerve terminal as a characteristic unit received support in an unexpected and different manner (Riker *et al.*, 1959). While analyzing the results of approximately 40 experiments in which 3-OH PTEA had been administered and motor nerve activity recorded, it was found that one third of the animals failed to exhibit repetitive antidromic spikes, regardless of the 3-OH PTEA dose. Despite this, electric repetition was always evident in the muscle. Furthermore, on the basis of our total experience it may be said that 3-OH PTEA will cause muscle potentiation regularly and predictably. The possibility remained that motor nerve damage occasionally prevented detection of the repetitive antidromic discharge. However, this was ruled out in most of the 40 experiments by finding that, when the motor nerve failed to display repetitive activity after 3-OH PTEA, it would react repetitively to 3-OH PTMA. While this indicates differences between the modes of action of 3-OH PTEA and 3-OH PTMA, the more important reference is physiological. In effect, 3-OH PTEA has dissociated orthodromic and antidromic repetition. This separation of forward and backward responses again points to the presynaptic terminal as a functional entity from which repetitive activity may originate.

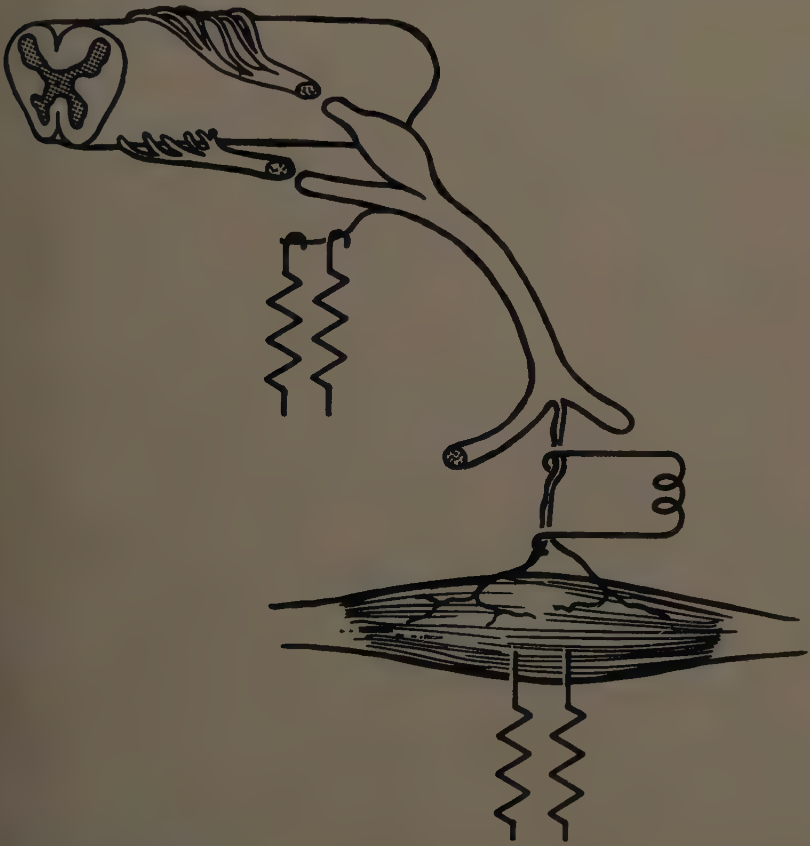

FIGURE 7. Scheme of experimental technique. Stimulating electrodes on nerve to gastrocnemius muscle. Recording electrodes on muscle and on ventral root filament (Riker *et al.*, 1959). Reproduced by permission from *The Journal of Pharmacology and Experimental Therapeutics.*

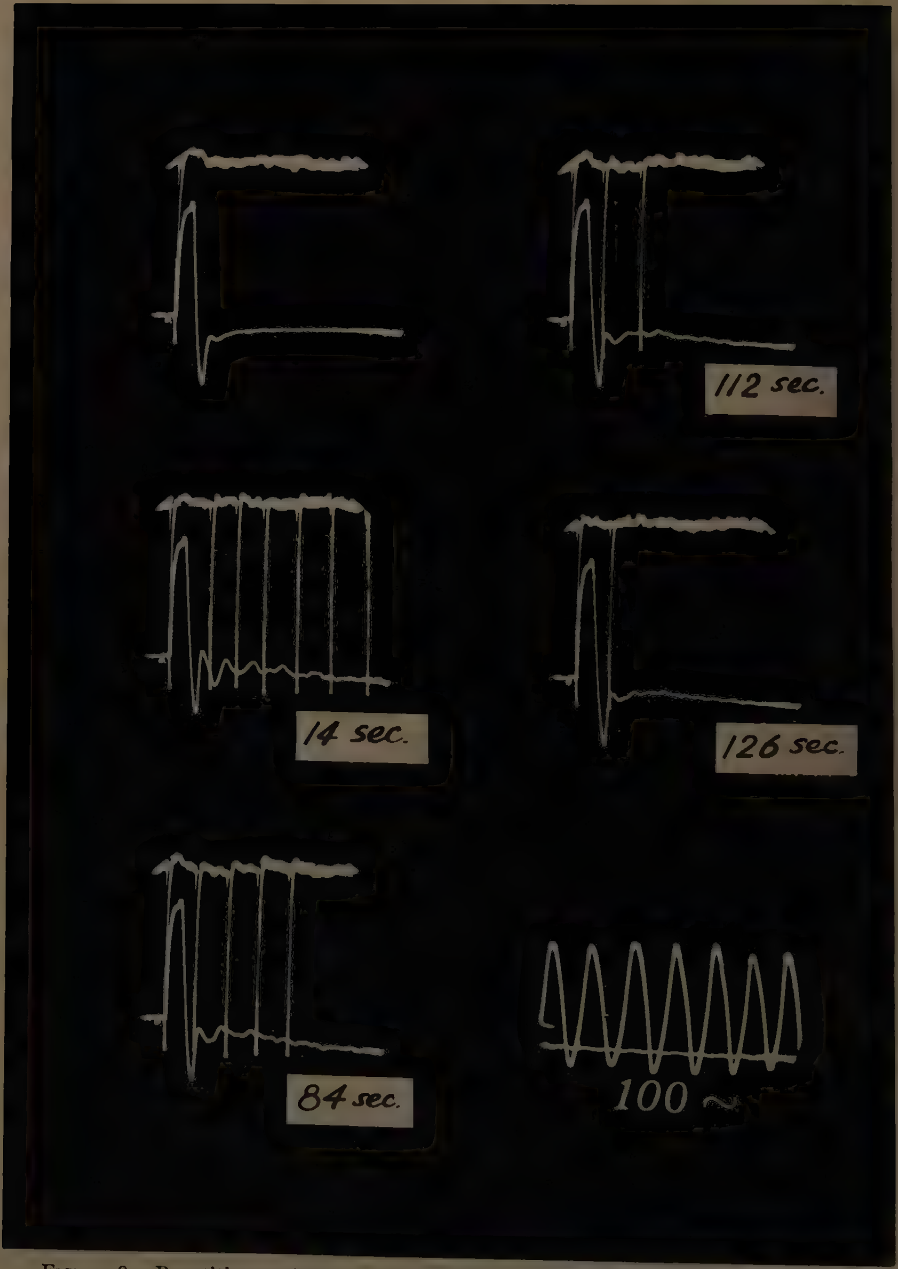

FIGURE 8. Repetitive antidromic discharge evoked by 25 μg./kg. 3-OH PTEA, intra-arterially in single ventral root fiber. Chloralose anesthesia (50 mg./kg. I.V.). The upper trace in each pair is the nerve activity recorded antidromically from the distal end of a severed L7 ventral root fiber; the lower trace is the muscle activity recorded with a needle electrode from the medial head of the gastrocnemius muscle. The nerve to the medial head was stimulated every 7 sec. with a square pulse of 0.5 msec. duration. The topmost pair of traces in the left column is the preinjection control. The stimulus-evoked muscle action potential and antidromic nerve spike are to the extreme left in each record. The negative variation in the nerve is downward, and that of the muscle is upward. Time in seconds after injection is indicated in the lower right corner of each record (Riker *et al.*, 1959). Reproduced by permission from *The Journal of Pharmacology and Experimental Therapeutics.*

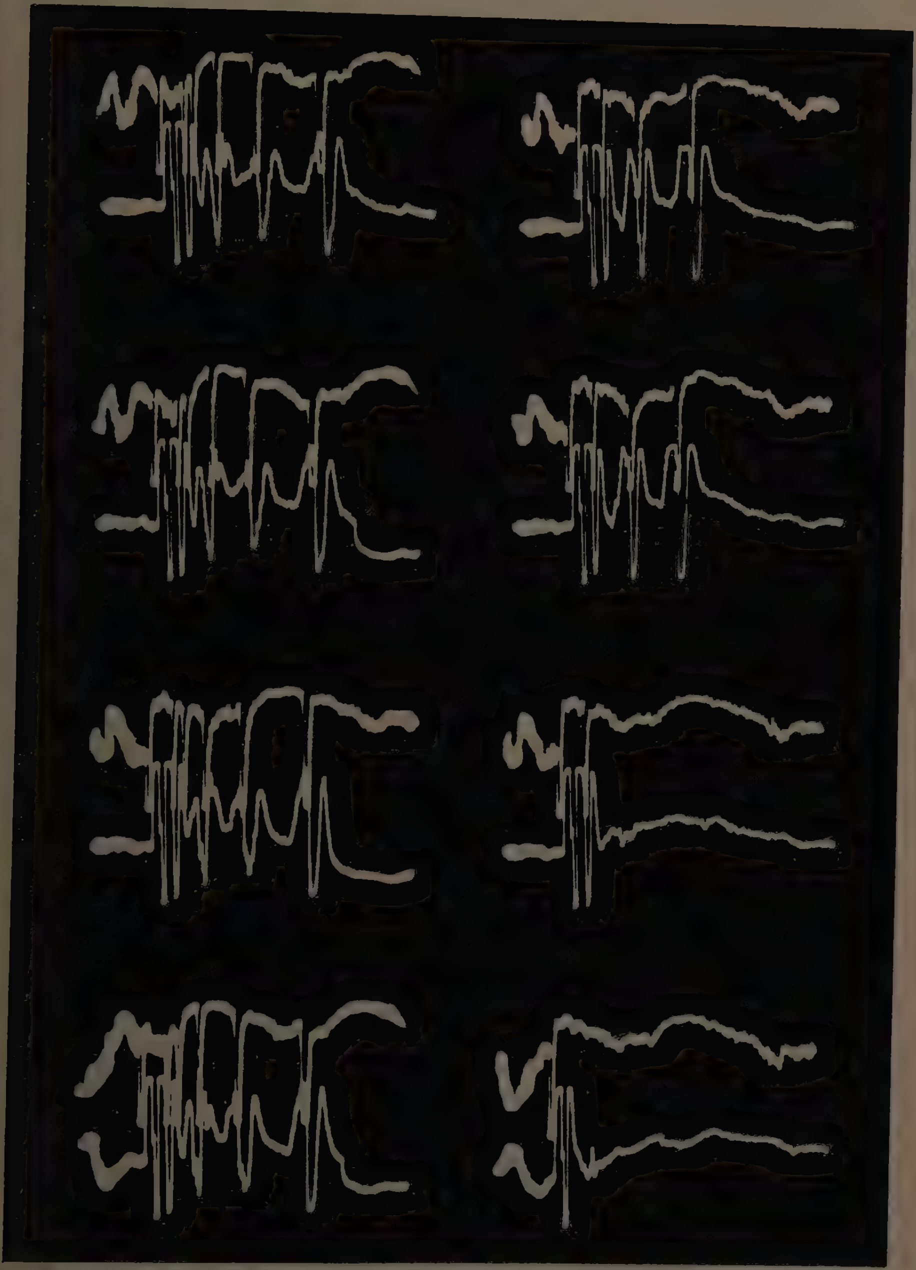

Figure 9. 3-OH PTEA-evoked repetitive discharges, appearing antidromically in a few ventral root fibers and orthodromically in the correspondingly innervated muscle fibers. Chloralose anesthesia (50 mg./kg. I.V.). The upper trace in each pair is the nerve activity recorded from the distal end of some L7 ventral root fibers; the lower trace is the muscle activity recorded with a needle electrode from the appropriate area of the medial head of the gastrocnemius muscle. The ventral root was stimulated every 7 sec. with a square wave pulse of 0.1-msec. duration. The topmost pair of traces in the left column occurs 5 sec. after the intra-arterial injection of 3-OH PTEA (200 μg./kg.). The remaining sweeps occur successively at intervals of 7 sec.

The motor nerve terminal as the site of 3-OH PTEA action and the functional activity of the presynaptic unit has also been recognized by pharmacological means (Riker *et al.*, 1959). Thus neurotropic depressants such as pentobarbital, cyclopropane, and procaine easily abolish the postactivation repetition that appears in motor nerve after 3-OH PTEA administration. This effect is shown for pentobarbital in FIGURE 10. The impressive point here is the fact that the nerve is stabilized by doses of pentobarbital in the sedative range (2 to 10 mg./kg. I.V.) and that these small doses usually have little effect on the muscle repetition. Therefore, this also serves to separate the forward and backward responses and thereby to delineate the motor nerve terminal. In conjunction with this, it is noteworthy that full anesthetic doses of pentobarbital only slightly diminish the twitch potentiation that is regularly produced by 3-OH PTEA. These doses of pentobarbital will prevent or abolish antidromic repetitive firing. From this it is concluded that orthodromic activation of muscle is less susceptible to depression of the presynaptic element than is antidromic activation of the axon. Furthermore, it may be pointed out that lethal doses of pentobarbital are without effect on the depolarizing activity of quaternary ammonium ions, as measured by the fibrillary discharge of chronically denervated cat muscle.

The effect of pentobarbital is reminiscent of the earlier findings by Brown (1937) and Bacq and Brown (1937) that ether and Dial inhibit the potentiation effect of physostigmine. In view of this and of the present work, it is proposed that the commonly employed anesthetic agents manifest their effects on neuromuscular transmission by an action on the motor nerve terminal. This accords with the views of Larrabee and Posternak (1952) for the effects of anesthetics on ganglionic transmission.

From the physiological standpoint, the separation of the forward and backward responses infers that multiplication of a single volley can occur in a structure intermediate to axon and end plate. Since the focus in question is obviously in functional continuity with the nerve, it is logical to assign this generator property to the motor nerve terminal.* The physiological counterpart of the pharmacological facilitation of neuromuscular transmission appears to be post-tetanic potentiation. In this respect, strong argument has been advanced to indicate that post-tetanic potentiation at the neuromuscular junction is attributable to a presynaptic effect (Hutter, 1952; Liley and North, 1953). The concept of the motor nerve terminal as a functional entity may be applied here, wherein its transmitter output in response to a given volley at any time will be determined by the immediate precedent activity that has traversed the pathway.

Previous work from this laboratory (Riker *et al.*, 1957) has already described the anticurare action of 3-OH PTEA. Certain characteristics of this action were noted to be consistent with the idea that the antagonism of curare by 3-OH PTEA comes about through the action of 3-OH PTEA on the motor nerve terminals. Thus, a nearly complete curariform transmission blockade is nearly but not fully counteracted by maximally effective doses of 3-OH PTEA.

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* This reference is made in the functional sense and does not have any morphologic correlation.

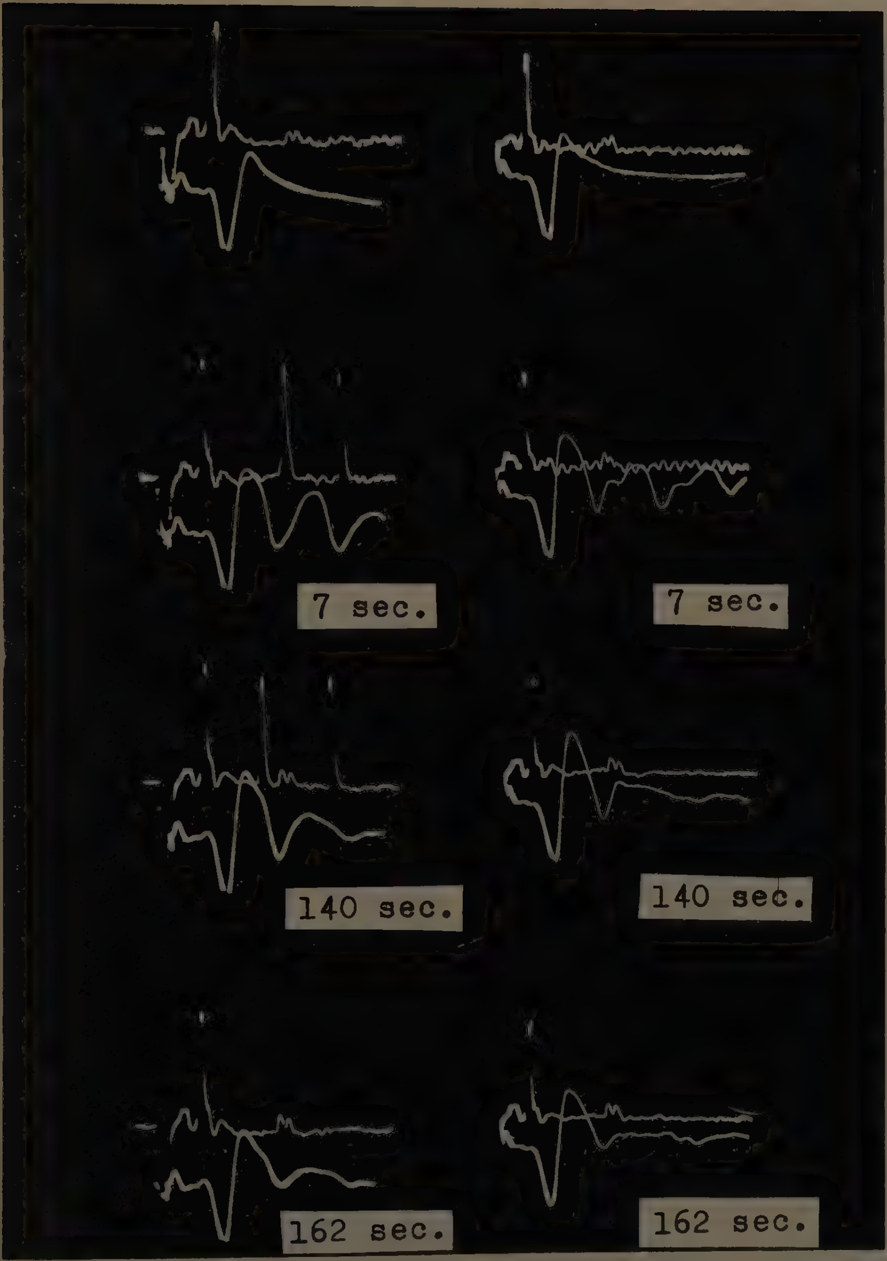

FIGURE 10. Dissociation by pentobarbital of nerve and muscle repetitive activity evoked by 3-OH PTEA in a cat anesthetized with chloralose (50 mg./kg.). The upper trace in each pair was recorded from the severed distal segment of an L7 ventral root filament containing few fibers. The lower trace is the muscle activity recorded from the medial head of the gastrocnemius. The negative variation in the muscle is downward. The nerve to the medial head of the gastrocnemius was stimulated every 7 sec. with a square wave pulse of 0.5-msec. duration. The negative variation in the nerve is upward. The left column shows the preinjection control (*top*). Subsequent records taken at times indicated after intra-arterial injection of 3-OH PTEA (50 μg./kg.). The right column shows the control record (*top*) taken 35 min. after the intravenous injection of 5 mg./kg. sodium pentobarbital; the subsequent records were taken at times indicated after intra-arterial injection of 3-OH PTEA (800 μg./kg.). From Riker *et al.*, 1959. Reproduced by permission from *The Journal of Pharmacology and Experimental Therapeutics.*

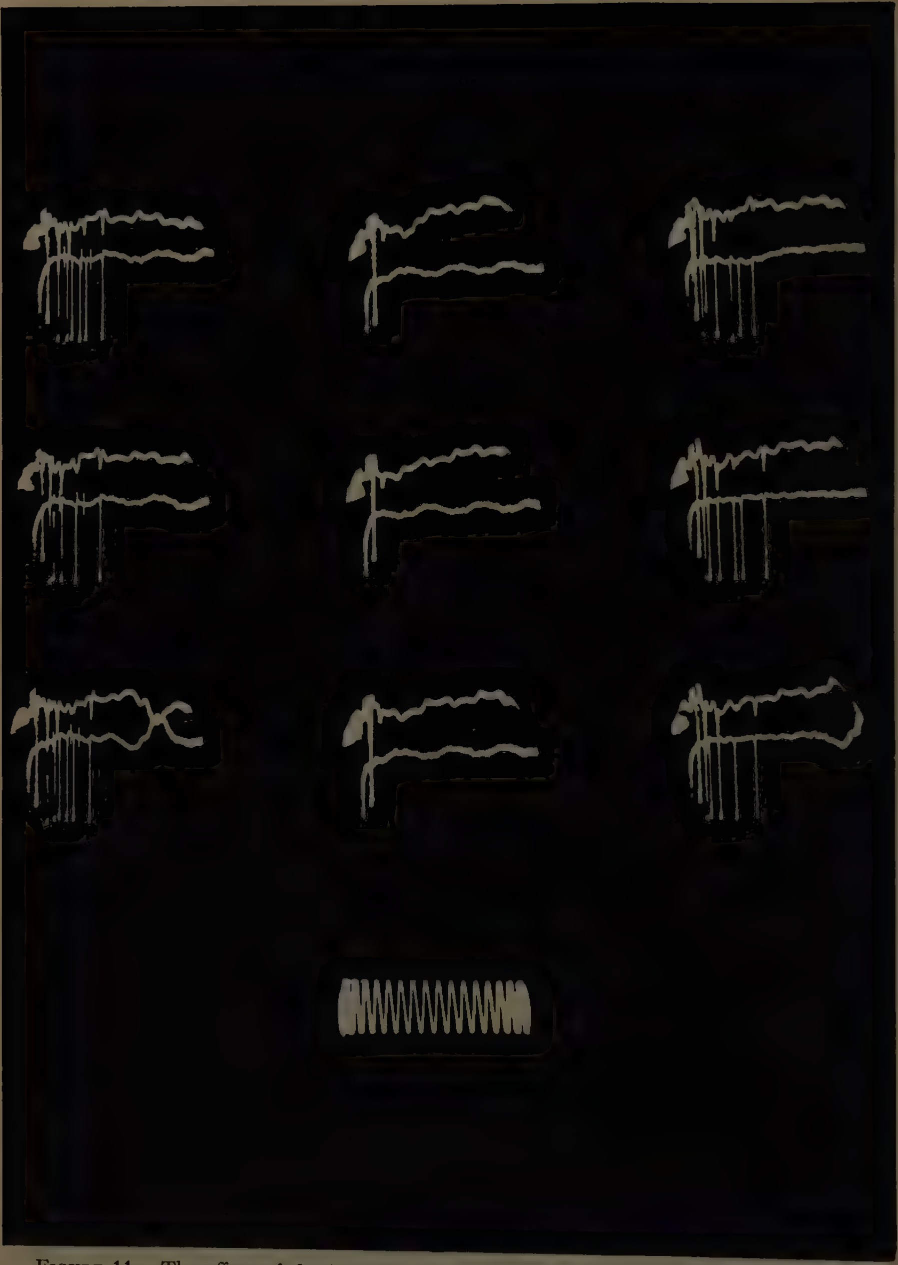

FIGURE 11. The effect of *d*-tubocurarine chloride on the antidromic repetitive discharge evoked in motor nerve by 3-OH PTEA. Chloralose anesthesia (60 mg./kg. I.V.). The upper trace in each pair is the muscle activity recorded with a needle electrode from the medial head of the gastrocnemius muscle; the lower trace is the nerve activity recorded from the distal end of a severed L7 ventral root fiber. The ventral root was stimulated every 7 sec. with a square wave pulse of 0.1-msec. duration. In each column the 3 sweeps depicted are those that immediately follow the injection of 3-OH PTEA (200 μg./kg.) intra-arterially. In the left hand column is the control response to 3-OH PTEA; the middle column shows the absence of response to 3-OH PTEA given 7 min. after the intra-arterial injection of 5 μg./kg. of *d*-tubocurarine chloride; the column at the right is the response to 3-OH PTEA 36 min. after the curare injection. Time signal: 250 cycles/sec.

This contrasts with the action of more potent anticurare substances, including 3-OH PTMA, Tensilon, and neostigmine, in which a directly acting component of drug action supplements the 3-OH PTEA-like effect. These effects together enable complete transmission restoration. It was concluded that the anticurare action of 3-OH PTEA is limited, since it depends for its expression on the effectiveness of presynaptic repetition. Moreover, it was pointed out that the effective anticurare dose range for 3-OH PTEA is identical to its effective potentiating dose range in the uncurarized preparation. This affirms the notion that both of these 3-OH PTEA effects are derived from the same mechanism of action.

In view of the above findings, the possibility has been considered that curare might exert an important effect on the motor nerve terminal. Earlier studies of Jaco and Wood (1944) made this alternative highly suspect when they found that curare, like procaine, can specifically abolish physostigmine potentiation without impairing transmission of single maximal nerve shocks. Recent experiments performed by us with the curariform drugs Flaxedil and *d*-tubocurarine have confirmed essentially the report of Jaco and Wood (1944) and further indicate that small doses of either drug, well below those that interfere with single-volley transmission, obliterate repetitive antidromic activity in the nerve (FIGURE 11). It appears that a principal action of curare occurs at the motor nerve terminal.

Summary

(1) It has been demonstrated that certain phenolic quaternary ammonium ions, prototypes of neostigmine action, facilitate neuromuscular transmission primarily by an action on the motor nerve terminal.

(2) In turn, the unique susceptibility of the motor nerve terminal to the facilitating drugs suggests for the presynaptic structure a functional identity that is distinct from the axon.

(3) The concept of a local presynaptic event in neuromuscular transmission is supported by those experiments that delineate the motor nerve terminal through dissociation of orthodromic postsynaptic responses and antidromic axonal responses triggered from the motor nerve terminal.

(4) The demonstration that the blocking action of curare occurs primarily at the motor nerve terminal accords well with the fact that the eminent anticurare agents are facilitating drugs that act at the motor nerve terminal.

(5) In the light of this new information on the site of action of curariform and anticurare drugs, and since these drugs have been instrumental in the analysis of the physiology of neuromuscular transmission, it is essential that our present version of this synapse be critically examined. The arrangement may be such that the two "all-or-none" elements, nerve and muscle, are related through the juxtaposition of presynaptic and postsynaptic units, each capable of graded and decremental activity.

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STUDIES ON THE MECHANISM OF CURARIZATION*

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During the past year, a group† in this laboratory has been studying the mechanism of curarization, using radioactively labeled true curares and, as biological structures, the electric organ of the electric eel *Electrophorus electricus*, the diaphragm of the rat, and the gastrocnemius muscle of the cat.

The electric organ was used as a result of previous experiments that had shown the action of true curares on the electric discharge. Albe-Fessard *et al.*[1] showed the disappearance of the indirect response of the organ mediated through nerve excitation with the use of true curares. The electric organ was also the object of a paper by Chagas and Albe-Fessard[2] in which these authors, working with live eels, showed the difference of action between true curares and acetylcholinelike or depolarizing agents. This work, done in Rio de Janeiro, was later confirmed and extended at Columbia University, New York, N. Y., by Nachmansohn *et al.*[3] In another paper, Chagas *et al.*[4] showed the existence of a time differential between the persistence in the effect of muscular curarization and the duration of inhibition of the electric discharge in the electric eel, the electric inhibition lasting for a much longer period than the muscular curarization. This pointed to the possibility of a recuperation of the electric organ from the curare bound by a cellular component.

This paper reports some experiments conducted with the electric eel *in vivo* and certain experiments *in vitro* having the object of identifying the component responsible for curare fixation, and other work extended to mammalian structures.

METHODS

The drugs used were the triethiodide of gallamine (TRIEG), dimethylisochondodendrine, and dimethyl-*d*-tubocurarine, labeled with C^{14} atoms and having a specific activity of about 1 μc./mg. TRIEG is a synthetic product prepared by Bovet and Lestrange,[5] and its radioactive form was obtained by introducing a C^{14} ethyl group during the last stage of ethylation. The isochondodendrine is extracted from the tropical vine *Chondodendrum platyphyllum* in an inactive form and methylated with 2 methyl radicals containing radioactive C^{14} at the phenolic oxygens in their molecules. A second iodomethylation is obtained by transforming its tertiary ammonium into a quaternary radical, a process that imparts biological activity to the molecule. The dimethyl-*d*-tubocurarine was prepared by methylation of the natural alkaloid with C^{14} methyl radicals.

All radioactivity was determined in an automatic gas-flow counter, and the number of counts per minute is referred to a layer of zero thickness to avoid the self-absorption effect and to allow the results to be proportional to the specific activity and comparable between them.

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† At this time this group is composed of myself, A. Hassón, E. Penna Franca, C. S. Crocker, L. Sollero, J. Braun Cantilo, and R. S. Moura.

Homogenization was done in a Waring Blendor and, more recently, in a Virtis homogenizer at 45 rpm.

Throughout this experimental series, dialysis was done in cellophane bags against chilled distilled water (4° C.), either continuously or frequently changed. A blank assay was done under conditions used during dialysis to determine the natural retention of curare in the bag. In this case a curare solution was put inside the bag and the residual radioactivity taken as background for count check.

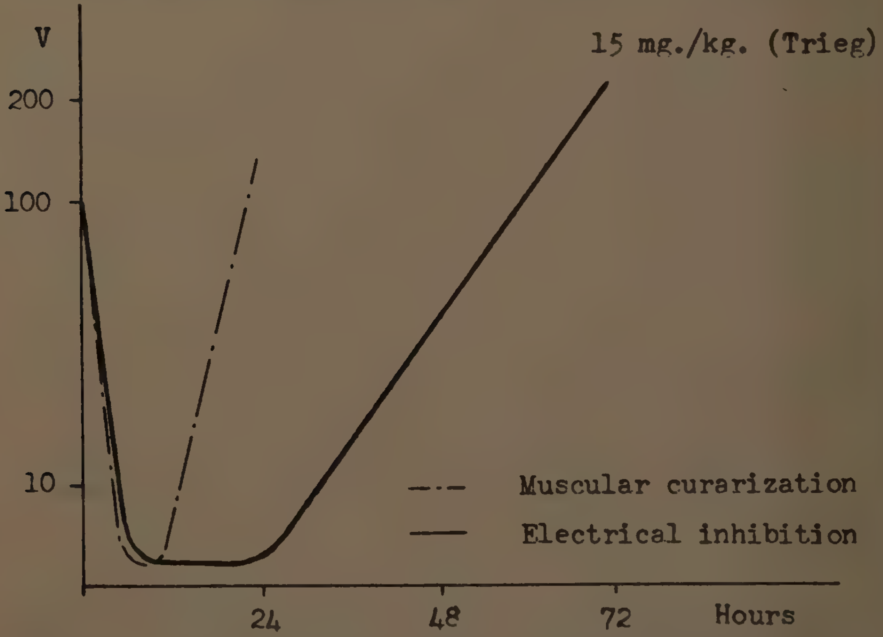

FIGURE 1. Graph of the curarization and electrical inhibition of *Electrophorus electricus*: abscissa, time in hours; ordinate, observed voltage (manual excitation).

In Vivo Experiments with the Electric Eel

An attempt was made to follow the fate of curare after its injection in the eel where muscular curarization and electric inhibition had occurred. To this end, curare was injected and the animal sacrificed during the stage of electric inhibition (see FIGURE 1) about 3 to 6 hours after injection. A saline perfusion was applied, using the appropriate Ringer's solution (Hargreaves and Frota Moreira[6]) to rid the organ of the nonfixed curare. The organ was then homogenized, treated with ether, and dialyzed against saline, as seen in FIGURE 2.

Results shown in TABLE 1 indicate that the curare forms a complex with a macromolecular constituent insoluble in ether and held in the aqueous phase. A series of physical techniques has been tried to determine the nature of this macromolecular complex. With the exception of the ultracentrifuge, none gave any promise. The most useful technique would appear to be electrophoresis, but it showed itself valueless, since even in a very weak ionic buffer solution the complex is dissociated by electric fields applied to it.

Some characteristics of this nondialyzable complex are: its lability in the presence of ionic solutions, its dissociation by all denaturing treatments of

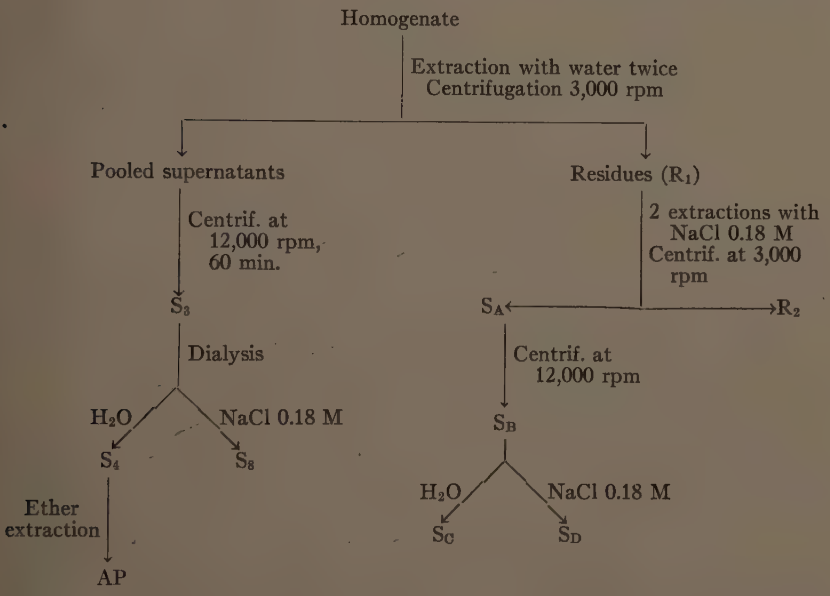

FIGURE 2. Schematic representation of the various procedures used to obtain the aqueous phase (AP).

TABLE 1

SPECIFIC ACTIVITIES OF THE DIFFERENT FRACTIONS OF ELECTRIC ORGAN HOMOGENATE AND 1 SAMPLE OF RAT DIAPHRAGM HOMOGENATE IN SOME OF THE EXPERIMENTS*

Experiment No.	Dose	Curarization time (hours)	Specific activity (cpm/100 mg. dry weight)									
			S_1	S_3	S_4	S_8	S_A	R_2	S_B	S_C	S_D	AP
4	15	3.0	64		109		61	10			15	
5	15	3.0	58	61	109	3	35	4	45	52	2	
6	15	10.0		124	186	9		14	105	153	15	174
7	50 (15 mg./kg. C^{14}-TRIEG)	9.0		200	281							302
9	40 (15 mg./kg. C^{14}-TRIEG)	8.5		106	145				12			156
19	40 (15 mg./kg. C^{14}-TRIEG)	6.0		97	170		70					142
25 (Rat diaph.)	20	0.5	139				279				9	327

* Data obtained from various steps in the preparation of the aqueous phase. Note that the specific activity is always greater in the aqueous phase.

proteins, and its displacement by molecules containing quaternary ammonium groups.[7] It seems that the curare recovered from the complex by denaturation has kept its chromatographic properties. It is thus reasonable to admit with these experiments as evidence, that curarization is due to the formation of a complex between the injected curare and a macromolecular component of the tissues.

Some kinetics aspects seem to point to a possibility that the curarization process may be the coupling of curare concentration at the fixing sites and its level in blood in the presence of the ionic concentration of plasma, the first controlling the dissociation of the complex, so that, as soon as curare concentration in the blood falls below the threshold value, decurarization begins to occur.

In Vitro Experiments with Electric Organ Extracts

After the failure of various experimental efforts to isolate the complex formed *in vivo* between the injected curare and the cellular component responsible for its fixation, experiments were undertaken *in vitro* using aqueous extracts of the electric organ to which curare was added. An aqueous phase was prepared in the same cycle of manipulation in which the aqueous phase containing the complex formed *in vivo* was obtained. When this was submitted to paper electrophoresis, the presence of various components was detected. Six components having a lesser mobility, similar to that of globulins, could be identified with bromphenol blue. Cholinesterase, present in the component of highest concentration, was determined by Koelle's reaction or by its activity after elution. Two other components were detected with Schiff's periodic acid reagent. These were stained by bromphenol blue and had a higher mobility than that of serum albumin. Another component, with a still higher mobility, could be colored by Morgan and Elson reagent for acetylhexosamine. This component, after elution, showed the greatest capacity for curare fixation, while that showing cholinesterase activity, after elution, showed a very small one.

Electrophoresis, using the Tiselius apparatus, in barbiturate buffer *p*H 8.6 and ionic strength 0.01 separated 6 components. The component with greatest mobility could be isolated from the upper part of the ascending branch of the double cell. This component showed a sixfold to eightfold increase in curare-binding capacity.

The chemical reactions of these components led to processes of purification that would yield glycoproteins or mucopolysaccharides.

Precipitation by trichloroacetic acid of the aqueous phase showed that the binding capacity was transferred to the supernatant (STCA). At the same time, alcoholic precipitation at varying *p*Hs showed an increase of the capacity to fix curare with increasing acidity up to *p*H 4; at this *p*H, fixation was at a maximum (TABLE 2).

Chemical analysis of this precipitate at *p*H 4 showed that an increase in hexosamine corresponded with an increase in capacity of fixation (TABLE 3).

The two techniques were then combined and alcoholic precipitation of STCA was tried. This alcoholic treatment gave an abundant precipitate that retained the binding power (TABLE 4).

When redissolved in an electrophoresis run at *p*H 8.6, this precipitate revealed the presence of two components: one neutral and one acid. The neutral component [f1 (FY)] constitutes approximately 73.2 per cent of the precipitate,

TABLE 2*

DATA SHOWING THE ALCOHOLIC FRACTIONATION OF THE AQUEOUS PHASE, VARYING THE *p*H AND THE SPECIFIC ACTIVITY OF THE VARIOUS REDISSOLVED PRECIPITATES

1. To AP(*p*H 7.0) add vol. of ethanol to 50 per cent (v/v) > PP 1 + SNT 1
2. SNT 1 + acetic acid to *p*H 6.0 > PP 2 + SNT 2
3. SNT 2 + ethanol 75 to 50 per cent > PP 3 + SNT 3
4. SNT 3 + acetic acid to *p*H 3.5 > PP 4 + SNT 4
5. SNT 4 evaporated and dialyzed/distilled water
6. SNT 5 conc. > *p*H 4 + 10 vols. ethanol > PP 5 + SNT 5

PP	Specific activity in percentages of original AP
II	113
III	70
IV	116
V	317

* Abbreviations used: AP = aqueous phase; PP = precipitate; SNT = supernatants.

TABLE 3*

INCREASE OF *N*-HEXOSE AND HEXOSAMINE CONTENT DURING PURIFICATION BY ALCOHOLIC PRECIPITATION

	AP (percentages)	PPV (percentages)
Nitrogen	11.0	4.8
Hexose	5.4	10.3
Hexosamine	2.4	6.4

* Abbreviations used: AP = aqueous phase; PPV = precipitate.

TABLE 4

FRACTIONATION OF STCA

ALCOHOLIC PURIFICATION OF THE TRICHLOROACETIC ACID PRECIPITATION OF THE AQUEOUS PHASE*

	Specific activity, cpm/mg.	
	Pure radioactive Flaxedil, 0.1 per cent	Flaxedil, μg./mg.
STCA from aqueous phase	7,000	21.8
STCA precipitate + 3 vol. ethanol (FY)	8,750	27
Supernatant of FY (FZ)	46,300	144

* The redissolved precipitate (FY) is composed of an active component common to the supernatant (FZ) and an inactive one (73 per cent).

and that having the acid characteristics [f3 (FY)] and mobility equal to 10.8×10^{-5} cm.^{2}/volt/sec., constitutes nearly 27 per cent. These components were then fractionated in a column of *N*,*N*-diethylaminoethylcellulose and were later analyzed. The fractionation showed that, as the capacity to bind curare is increased, there is also an increase in nitrogen, uronic acid, hexosamine, and acetylhexosamine content in relation to the nonpurified solution, as seen in TABLE 5.

The acid fraction thus obtained takes strong metachromatic coloration in paper electrophoresis.

Mucopolysaccharides were extracted directly from the electric organ by Dorfman and Cifonelli's technique[8] for the preparation of acetone powder, and the solution obtained by saline extraction of this powder was run in a column of cellulose, as described by those authors. The results show that the binding of curare on the fraction thus obtained is of the same order of magnitude as found in the fraction collected after column separation of the alcoholic precipitate of STCA, redissolved, and rerun in a column of *N*,*N*-diethylaminoethylcellulose. A comparison between the binding power of these purified extracts and various other macromolecules is presented in TABLE 6.

Table 5

Fractionation and Chemical Analysis of FY (STCA) in an *N*,*N*-Diethylaminoethylcellulose Column*

	Flaxedil fixation		Nitrogen (percentages)	Uronic acid (percentages)	Hexosamine (percentages)	Acetylhexosamine (percentages)
	cpm/mg.	µg./mg.				
FY (STCA)	12,000	73.0	1.43	7	7.0	6.8
f1 (FY)	245	1.6	0.00	0	0.0	0.0
f3 (FY)	43,000	270.0	4.20	20	23.5	25.0

* The active component (27 per cent) retains all of the binding.

Table 6*

Comparison of Binding of TRIEG by Various Fractions of STCA of the Electric Organ and Other Macromolecules

Material	TRIEG binding (µg./mg.)	Acetylhexosamine (mg. per cent)	Uronic acid (mg. per cent)
PPT 2	98.9	17.0	10.1
PPT 3	107.0	15.0	9.0
FY	73.0	6.8	7.0
f3 (FY)	270.0	25.0	22.6
Potassium hyaluronate	300.0	23.3	27.5
Chondroitin sulfate A	493.0	30.0	28.7
Chondroitin sulfate B	356.0	—	—
Chondroitin sulfate C	238.0	—	—
Heparitin sulfate	353.0	—	—
Kerate sulfate[11]	344.0	—	—
STCA, human plasma	81.0	7.85	0
Acetylcholinesterase†	0.73	0	0

* Abbreviations: PPT 2 and PPT 3 are the redissolved precipitates of an acetone powder of the electric organ; FY is the redissolved precipitate of STCA; and f3 (FY) is the active fraction of FY collected from *N*,*N*-diethylaminoethylcellulose.

† The acetylcholinesterase extracted from the electric organ was prepared by the method of Hargreaves[10] with a two hundredfold increase in enzymatic activity.

The binding capacity of the supernatant of trichloroacetic precipitation of human plasma is included in the above table. It was prepared following the technique of Vaux St. Cyr *et al.*[9] and consists of precipitation by acid alcohol and yields a precipitate containing 7.85 per cent acetylhexosamine, 10.6 hexosamine, and cyolic acid. This precipitate shows, in paper electrophoresis at *p*H 8.6, a single component reacting to bromphenol blue and having Schiff's coloration.

These results led us to believe that, in the aqueous phase of the electric organs, acid mucopolysaccharides are the substances responsible for curare binding; all of the experimental indications point to this conclusion. This does not postulate that such substances participate in the phenomena of transmission. It has yet to be determined whether the observed experimental evidence is due to a coincidence alone attributable to adequate chemical constitution, observable only *in vitro*, or whether these substances play any role in such a complex mechanism as neuroeffector transmission. Speculation on this point will possibly lead to the admission of a distinction between certain specific receptors responsible for specific biological actions located in certain regions of the tissues and nonspecific receptors without widespread biological consequences.

In this paper, nevertheless, it seems worthwhile to indicate that, upon the occurrence of curarization, the substances that constitute the ground substance must bind a certain number of molecules of the injected drug. As a matter of fact, this can well be seen in the electric organ by autoradiographic techniques that reveal that the curare is fixed, not only at the synaptical posterior surface of the electroplax, but also at the anterior one and, further on, by the fact that in other animals curare is found fixed in various organs even after recuperation from curarization.

One can support the thesis that the duration of curarizing phenomena seen in the electric organ, as observed by Chagas *et al.*[4] is due to the fact that the curare forms a complex with a receptor existing in the rostral surface of the synaptic regions precisely at the site of production of inhibition of nervous transmission. As the curare will be eliminated from those points by competition with the chemical transmitter, diffusion from the curare bound by mucopolysaccharides will occur and renew the complex at the synaptic region, and thus prolong the duration of curarization.

However, much work must yet be done in order to correlate the findings obtained from the *in vitro* experiments and the *in vivo* observations.

EXTENSION OF THE WORK TO OTHER MAMMALIAN STRUCTURES

After the beginning of this work it was seen that in curarized rats the diaphragm treated in the same way as the electric organ yielded a complex having the same chemical properties as those from the electric organ. The results of a series of experiments made with mammalian structures are presented in TABLE 7.

In this table the first column shows the results obtained in experiments *in vitro*, and when a complex between the STCA obtained from the aqueous phase and TRIEG was formed. The experiments listed in the second column were done *in vivo* with rabbits, and the curare employed was dimethyl-*d*-isochondodendrine. The values of the *in vitro* experiments (column 1) are consistently very much higher than those found in the *in vivo* experiments (column 2); the difference is due to the differing conditions operating during the two experiments. In the *in vitro* reaction, the curare and the receptor are mixed under saturation concentration and the drug is bound by any kind of receptor liberated by homogenization of the organ. In the *in vivo*, only a small number of receptors binds the curare. It is probable that part of the complex formed is dissociated by the perfusion of the organ done in order to rid the various organs from the nonfixed curare. It should also be pointed out that in both experiments the sympathetic ganglion chain was not dissected from its connective tissue. It is to this fact that we attribute the higher specific activity existing in the cervical ganglion that was dissected, as compared to the sympathetic chain. Where the adrenals are involved, no separations of the cortex and the medulla are made. Dissection of the atrial part of the auricles was also performed in a nonselective way. From the observations recorded in the first column of TABLE 7 one may conclude that all animals have a fairly large capacity to bind molecules containing quaternary ammonium radicals, although there seems to be a more active binding in structures that are rich in neurovegetative synapses. This is strongly supported by the data obtained from the sympathetic chain, superior cervical ganglia, and the adrenals, and in the differences observed between the central and peripheral diaphragm and between the auricle and the ventricle. Complex formation, attempted under the same conditions but using isolated adrenal medulla and cortex, showed that there exists a two- to fourfold difference in binding power between the medullar and cortical extracts, the former being the more active.

TABLE 7

Organ	*In vitro*, dogs, 5 (gm./gm. tissue)	*In vivo*, rabbits, 8 (gm./gm. tissue mg. inj./kg. animal)
Adrenals	346.0	0.222
Sympathetic chain	389.0	0.309
Superior cervical ganglion	875.0	
Diaphragm, center	96.5	0.018
Diaphragm, periphery	216.0	0.039
Auricle	185.0	0.373
Ventricle	126.0	0.140
Muscle	89.0	0.026
Frontal lobe	132.0	
Lateral lobe	63.0	
Base nuclei	95.5	
Liver	88.0	0.428
Kidney	110.0	0.595

On analyzing the results presented in TABLE 8 one supposes that, in the experiments done *in vitro*, curare is fixed not only by receptors of biological specificity, but also by chemical substances that are not reached by the drug *in vivo* and are able to form complexes only after homogenization. Those substances could be considered as nonspecific receptors. Specific receptors may be termed those occupying certain regions of the tissues and having special characteristics.

Another speculative direction is to consider that the receptor is of the same molecular species, biological specificity being due to what could be termed a "sitic" specificity; that is, pertaining to the localization of the substance in the sites where certain biological phenomena may occur.

Curare Fixation in Denervated Structures

The binding power of curare is partially lost in denervated electric organs, as shown in TABLE 9.

This finding was not confirmed in preliminary experiments done with mammalian muscle. The first series, in which the diaphragm of the rat was used, the right side being phrenicectomized by transthoracic operation, showed an increase of complex formation on the denervated side. TABLES 10 and 11 summarize the data.

TABLE 8

DATA OF THE BINDING POWER *IN VITRO* AND THE COMPLEX FORMATION *IN VIVO* IN 2 SERIES OF EXPERIMENTS

Experiment No. 5 (*in vitro*)

		µg./mg. dry weight	µg./gm. tissue
AP	Cortex	1.23	7.82
AP	Medulla	5.79	79.73
AP	Adrenal	2.13	18.94
STCA	Cortex	42.84	14.60
STCA	Medulla	74.28	26.54
STCA	Adrenal	60.11	20.12

Experiment	5	6	7		8	
Binding relation medulla/cortex	4.7 TRIEG	12. TRIEG	4.2 TRIEG	2.6 Iso.	3.7 TRIEG	2.4 Iso.

Experiment No. 13 (*in vivo*)

µmoles/mg.

		TRIEG	Iso.-C^{14}
AP	Cortex	0.0009	0.0006
AP	Medulla	0.0032	0.0016
AP	Adrenal	0.0014	0.0008

It must be emphasized, as shown in TABLE 11, that when the animals were treated with a prior injection of a depolarizing agent, depolarization of the receptors produced a great reduction in complex formation.

In the second series of experiments, curare was injected in a close arterial injection in the denervated or nondenervated gastrocnemius of the cat. The amount of curare fixed was determined by subtracting from the injected quantity of the drug the amount recovered from the blood flowing from the muscle. Circulation conditions were kept constant to the degree possible. The results are presented in TABLE 12.

TABLE 9

RESULTS OF CURARE FIXATION IN DENERVATED ELECTRICAL ORGANS*

Days	Experimental conditions	Drug used	Cpm at zero thickness per 100 mg. dry weight			Fixation (percentages)	Cholinesterase		
			CL	D	N		CL	D	N
First series of experiments									
4	*In vivo*	TRIEG	2,605	1,878		72	100	108	
5	*In vivo*	Iso.-C^{14}	992	494		40.8			
21	*In vivo*	TRIEG	1,472	627		22.2	100	39	
22	*In vivo*	TRIEG	11,082	9,083		82	100	49	
28	*In vivo*	TRIEG	3,490	1,830		52.5	100	32	
36	*In vivo*	Iso.-C^{14}	2,615	1,363		52.1			
15	*In vitro*	TRIEG	2,017	1,822		90.3			
33	*In vitro*	Iso.-C^{14}	1,285	900		71.7			
Second series of experiments									
2	*In vivo*	Iso.-C^{14}	3,450	3,200	2,930	92.7	100	116	77
4	*In vivo*	Iso.-C^{14}	2,950	2,460	2,680	83			
8	*In vivo*	Iso.-C^{14}	1,280	800	1,350	62	100	110	79.5
16	*In vivo*	Iso.-C^{14}	5,900	4,800	5,000	81	100	80.4	73.8
2	*In vitro*	Iso.-C^{14}	4,800	3,800	4,750	80.5			
4	*In vitro*	Iso.-C^{14}	12,500	7,400	11,900	75.0			
8	*In vitro*	Iso.-C^{14}	17,500	13,200	17,000	70			

* The first column indicates the number of days elapsed since denervation; CL means contralateral in denervated sides to be compared against the denervated one (D); N indicates the organ removed outside the region of denervation. In the first series the eels were operated upon on one side, and in the second series the incision was made on both sides, but only one side was denervated.

TABLE 10

COMPARATIVE DATA BETWEEN THE LEFT (DENERVATED) SIDE OF THE DIAPHRAGM OF THE RAT AND THE RIGHT (NORMAL) ONE

Rat No.	Side of diaphragm†	cpm/gm./min.‡
1	Left	6,110
	Right	3,508
2	Left	3,572
	Right	1,989
3	Left	3,280
	Right	1,378
4	Left	4,780
	Right	2,688
5	Left	8,750
	Right	4,170
6	Left	5,600
	Right	4,600
7	Left	8,317
	Right	4,226

* Rats of the same age and weight were utilized.

† Averages: denervated hemidiaphragm (L), 17.7 μg./gm.; normal hemidiaphragm (R), 8.9 μg./gm.

‡ TRIEG was used; an average dose of 5 mg. was administered.

The results show a lesser fixation in normal muscle as compared to the denervated one. Both series of experiments are subject to criticism. In the case of the diaphragm, TRIEG was the drug used; it is relatively weak in action, so that a high concentration of the compound was needed.

TABLE 11

COMPARATIVE DATA BETWEEN THE LEFT (DENERVATED) SIDE OF THE DIAPHRAGM OF THE RAT AND THE RIGHT (NORMAL) ONE*

Rat No.	Side of diaphragm†	cpm/gm./min.
12	Total	1,654
13	Left	1,495
	Right	794
14	Left	1,524
	Right	375
15	Left	1,488
	Right	858
16	Left	14,815
	Right	461
17	Left	1,236
	Right	348
18	Left	1,450
	Right	651
19	Left	1,186
	Right	325

* Rats injected previously with succinylcholine.

† Averages: denervated hemidiaphragm (L), 17.7 μg./gm.; normal hemidiaphragm (R), 8.9 μg./gm.

TABLE 12

COMPARATIVE DATA ON THE BINDING OF CURARE (TRIEG) IN THE NORMAL AND DENERVATED* GASTROCNEMIUS OF THE CAT†

Expt.	Mg. curare per gm. tissue		Relations	
	Normal	Denervated	Fixation N/D‡	Weight N/D
4	0.53	0.95	0.55	1.8
5	0.74	1.1	0.67	1.3
6	0.55	1.1	0.50	1.8

* Denervation of 21 to 24 days.

† The figures represent the difference in injected curare and the amount remaining in blood drawn from the muscle.

‡ Normal/denervated.

In the second series of experiments the fate of the total injected curare could not be ascertained, as experimental conditions did not ensure a perfect balance between the injected and the recovered drug. Thus, the data obtained may not be more complete than those that represent the curare that was actually fixed.

If these differences are confirmed in future experiments, they could be interpreted as showing that denervation produces the disappearance of acetylcholine liberation and eliminates competition between the transmitter and the drug, thus allowing for a better fixation of the latter. Such conclusions should nevertheless be appraised with great care, because we do not know exactly what happens to the metabolism of a cellular structure after denervation, particularly with respect to the receptors. Thus, no definite conclusion can be reached as yet.

These considerations, while valid for mammalian muscle, are still more pertinent to the electric organ, where the experimental evidence points to an appreciable diminution of curare binding in denervated organs, and where the existence of specific and nonspecific receptors seems to be well established.

CONCLUSIONS

(1) *In vivo* experiments undertaken with the electric organ of the *Electrophorus electricus* indicate that the phenomenon of curarization is due to the formation of a complex between the injected drug and a still unidentified cellular macromolecular component. This complex is labile, and it seems that curarization is the result of relative curare concentration level and ionic concentration in the blood.

(2) In aqueous extracts of electric organ of *Electrophorus electricus* the substances that seem to be responsible for curare fixation are the acid mucopolysaccharides. The aqueous extracts of various mammalian organs show that there is a gross relation between the concentration of these substances, synaptic abundance, and the ability to bind curare.

(3) One method of interpreting certain of the findings would be to consider the existence of specific receptors that correspond to special molecular species existing in certain regions and biochemical receptors able to fix curares that are widely distributed along the tissues with no evident specific consequent biological action. It is pointed out that one could consider the existence of specificity of sites by which the substances would play a role in the biological phenomena merely by being present in certain regions of the tissue where they occur.

(4) The data related to denervated structures appear to be contradictory. They could have an important significance in the general interpretation of transmission phenomena. There is strong experimental evidence that there is a diminution of curare binding in the denervated electric organ, where specific and nonspecific receptors have been demonstrated. The experimental findings in mammalian muscle showing a greater fixation capacity in denervated muscle are not yet conclusive.

ACKNOWLEDGMENTS

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Part III. Sensory Receptors

THE NERVOUS MECHANISM OF TASTE

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Electrophysiological studies of the impulse traffic in the taste nerves have in general confirmed the old conception that there are four classes of taste, namely, sweet, sour, bitter, and salty. The response of single taste fibers in amphibians as well as in mammals has revealed that the taste fibers seem, to a great extent, specific. A complication was added to this view, however, when Pfaffmann (1941) found in the cat that strong acid solutions not only stimulated specific "acid fibers," but also "salt fibers." The salty taste would thus be discriminated from acid taste only by the absence of impulses in the specific acid-taste fibers.

A further complication in the classic psychophysical way of regarding the matter was the finding that the frog possessed taste fibers that respond to the application of pure water to the tongue (Zotterman, 1949, 1950). I originally believed that the fibers that responded specifically to water (Andersson and Zotterman, 1950) served a particular purpose in the regulation of the water intake in these animals, which live chiefly in fresh water. This finding, however, raised the old question, debated by psychologists for a half century at least, of whether mammals, including man, are equipped with specific taste organs for water. At Stockholm University, David Katz had long maintained that this must be the case. In examinations he often put the question: "What is the taste of water?" The correct answer was: "wet."

In 1954 Liljestrand, confirming earlier investigations, found in experiments upon himself that the threshold value for NaCl solutions lay between 0.009 and 0.002 M, that is, about 0.05 to 0.01 per cent NaCl. This was just between the concentrations at which I had found that the specific water taste fibers began to respond. Liljestrand also found that Stockholm tap water (with a dry residue of 0.014 per cent) could be distinguished from distilled water (dry residue of 0.0004 per cent) with a certainty of 100 per cent. This also held after the tap water had been boiled for 5 minutes and then cooled (Liljestrand and Zotterman, 1954). In the old literature the flat taste of water was held to represent a special *sapor insipidus* or *sapor aquosus* (see Öhrwall, 1891). According to Henle, 1880, this would be characteristic of solutions containing less salt than the saliva; Henle maintained that the flat taste is to the sense of taste what black is to the sense of vision. An alternative interpretation was suggested, however, by the experiments on water taste in the frog that were quoted above. Furthermore, Skramlik (1926) reported that in humans salt solutions below 0.03 M taste sweet, and that saltiness appears only with concentrations above 0.03 M. For that reason, in 1954, Liljestrand and I decided to study the effect of water upon the taste fibers of the chorda tympani in some mammals.

Water Fibers in Mammals

Beginning with the action potentials from the entire chorda tympani nerve in the cat, dog, and pig, we were able to demonstrate that these animals responded positively to the application of water to the tongue. By dividing the nerve into fine strands we were able to find preparations that responded to the application of water to the tongue but not to 0.5 M NaCl solution, and also strands that responded to 0.5 M NaCl but not to water. In a further investigation on single taste fibers from the chorda tympani of the cat, Cohen, Hagiwara and I (1955) found that the activity of the water fibers was depressed by various inorganic salt solutions with a concentration above 0.03 M. Some, but not all, of the water-taste fibers were found to be stimulated by quinine as well as by acids of *p*H below 2.5. In addition to this, fibers were found that responded only to quinine but not to water or salt and only to a very small degree to acids. In confirmation of Pfaffmann's findings (1941) many salt-taste fibers in the cat responded to acids, as well. No fibers were found to respond specifically to sucrose or other substances that taste sweet to humans. Sucrose dissolved in water did not inhibit the response of the water fibers.

As will be seen from TABLE 1, there are only 2 test solutions that in the cat stimulate only one kind of taste fiber: water and NaCl. It is interesting to note that NaCl is the only salt which in humans elicits a pure salty sensation.

TABLE 1
FIBER TYPE RESPONSE

Stimulus	"Water" fiber	"Salt" fiber	"Acid" fiber	"Quinine" fiber	Sensation evoked
H_2O (salt <0.03 M)	+	0	0	0	Water
NaCl (>0.05 M)	0	+	0	0	Salt
HCl ($>p$H 2.5)	+	+	+	0	Sour
Quinine	+	0	0	+	Bitter

That the cat does not possess any fibers that respond to sweet-tasting substances such as sucrose (Zotterman, 1935) was confirmed by Pfaffmann in 1941. Recent investigations have also revealed that the calf and the lamb either lack "sweet" fibers in their chorda tympani or have very few of them. Sweet-tasting solutions produce positive responses in the rat, the dog, and the pig, but a very sweet-tasting saccharin solution, 0.02 M, fails to produce any response in these animals. In contrast to the mammals previously tested, the rat did not give any positive response to water. The application of water to the rat's tongue caused only an immediate decrease in the spontaneous activity that could be reduced to nil, returning slowly within 10 seconds of the cessation of the water flow. After a water rinse, even as weak a solution as 0.003 M NaCl caused an obvious and persisting response in this animal. The high level of spontaneous activity in the rat's chorda tympani is most probably an expression of the high sensitivity of its taste receptors to NaCl. In other mammals, such as the cat, the dog, and the pig, the salt receptors seem to adapt completely and quickly to a 0.1 M NaCl solution. After a previous rinse of water, however, there occurs a transient phasic response of salt-taste fibers of the cat to as weak a solution as 0.002 M NaCl.

The very high sensitivity of the rat to weak NaCl solutions found in these experiments (Zotterman, 1956) accords with Pfaffmann's experiments on rats and can in itself explain the high discriminatory ability of these animals. There is as yet no information in regard to salt-discrimination in the cat, dog, or pig. If these animals were entirely dependent upon their salt-taste fibers, their discrimination of salty solutions would be rather poor, but these species possess water-taste fibers that may play a part in discrimination between pure water and weak salt solutions.

Taste Fibers of Birds

Electrophysiological investigations of taste in birds have been made recently by Kitchell and myself (1959) and to date have been done only on chickens and pigeons. In the chicken, taste fibers were found in the glossopharyngeal nerve, which run both to the tongue and to the pharynx. Responses were observed following the application of distilled water, salt, glycerine, ethylene glycol, quinine, and acetic acid solutions to the tongue. Sucrose and saccharin solutions did not produce any positive response in the chicken.

Table 2

Comparison of Taste and Behavioral Response in Chickens and Pigeons

	Chicken	Pigeon
NaCl	TR +	TR +
	BR R*†	BR R*
Sucrose	TR −	TR −
	BR P*†‡	BR A*
Glycerin	TR +	TR +
	BR R*	BR R*
Ethylene glycol	TR +	TR +
	BR 0	BR 0
Saccharin	TR −	TR +
	BR R*†‡	BR R*
Quinine	TR +	TR −
	BR R*†	BR A*
Acetic acid	TR +	TR +
	BR R*	BR R*

Key to symbols: TR, electric response from nerves; BR, behavioral response according to *Engelmann, 1934; †Kare *et al.*, 1957, or ‡Jacobs and Scott, 1957. R, reject; A, do not discriminate against; P, prefer; and 0, not determined.

In the pigeon, also, there was a strong response to distilled water as well as to salt, glycerine, ethylene glycol, and acetic acid, but no response to sucrose. In about one half of the number of pigeons investigated, however, we noticed a positive response to the application of saccharin. No response was observed to quinine. Both species have numerous specific cold-responding fibers ending in the tongue, but no receptors were found that responded to warming the tongue until the tongue temperature was raised above 45° C. This indicated the absence of specific warm receptors and suggested that the activity observed was due to stimulation of nerve endings subserving pain.

Our findings are in good general agreement with the studies of the behavior of the bird in relation to a particular substance, as may be seen from TABLE 2. In two instances, however, there appears to be a difference in the results. Chickens seem to prefer sucrose solutions to water (Engelmann, 1934; Kare *et al.*, 1957; Jacobs and Scott, 1957), whereas no response to sucrose was observed in our studies. The degree of preference observed in the behavioral studies was small in all instances. Jacobs and Scott suggested that the preference could be due to a difference in viscosity rather than to a taste response. The results of behavioral studies show that chickens prefer water to saccharin, while in our experiments no taste response to saccharin in Ringer's solution was observed. Saccharin in distilled water produced a response indistinguishable from that to distilled water alone. Our observations do not suggest the possible sources of the afferent flow that enables chickens to discriminate against saccharin, but we can state that it probably does not originate from any taste receptors in the tongue.

It appears inexplicable that in our experiments 50 per cent of the pigeons responded positively to saccharin although they all lacked fibers responding to sucrose and quinine. In behavioral tests the pigeons reject saccharin, but do not discriminate between quinine solutions and pure water. For this reason I venture seriously to suggest that saccharin may taste salty to these birds.

Taste Fibers in the Monkey

In an uncompleted series of experiments on the rhesus monkey very strong responses were recorded from the chorda tympani on the application of water to the tongue, as will be seen from FIGURE 1. After previously rinsing the tongue with Ringer's solution the response to Ringer's solution is very small, while after a rinse with water there is a transient response (FIGURE 1). It will be seen from this figure that the integrated response to 0.5 M NaCl solution is smaller than that recorded after the application of an equal amount of distilled water. The relation between the water and salt response varies individually; this does not necessarily depend upon the relative number of specific fibers in action, but may depend on the size of the fibers, as the recorded spike height varies with the diameter of the fiber. A quantitative comparison of the integrated responses is therefore not a safe method of ascertaining the relative numbers of the various specific fibers unless their fiber size is known. FIGURE 2 shows some records led off from a fine strand of the chorda. This preparation contained one fiber that produced fairly large spikes when NaCl was applied to the monkey's tongue. The spontaneous activity of this salt fiber, which produced large spikes, was suppressed by the application of distilled water. When the tongue was irrigated with sucrose, saccharin, glycerine, or quinine solutions, a very massive response of smaller spikes appeared.

In FIGURE 3, records are presented from another preparation of the same chorda tympani that responded vigorously to salt but not to sugar or quinine. The threshold of the receptor of this fiber lay somewhere between 0.02 and 0.01 M NaCl. This particular salt-taste fiber also responded, but to a slight degree, to 0.2 M acetic acid. In later experiments, however, we have found several single salt-taste fibers that did not respond at all to an acid stimulus.

The chorda tympani of the monkey apparently contains a large number of fibers that respond specifically to sweet-tasting substances such as sucrose, glycerol, ethylene glycol, and even to saccharin. To date the monkey has been the first animal possessing specific fibers responding to sucrose in which

I have found that these fibers also respond positively to saccharin. In dogs, pigs, and rats, all animals which possess specific sweet-taste fibers, saccharin does not produce any positive effect on the sweet receptors. In the dog, strong

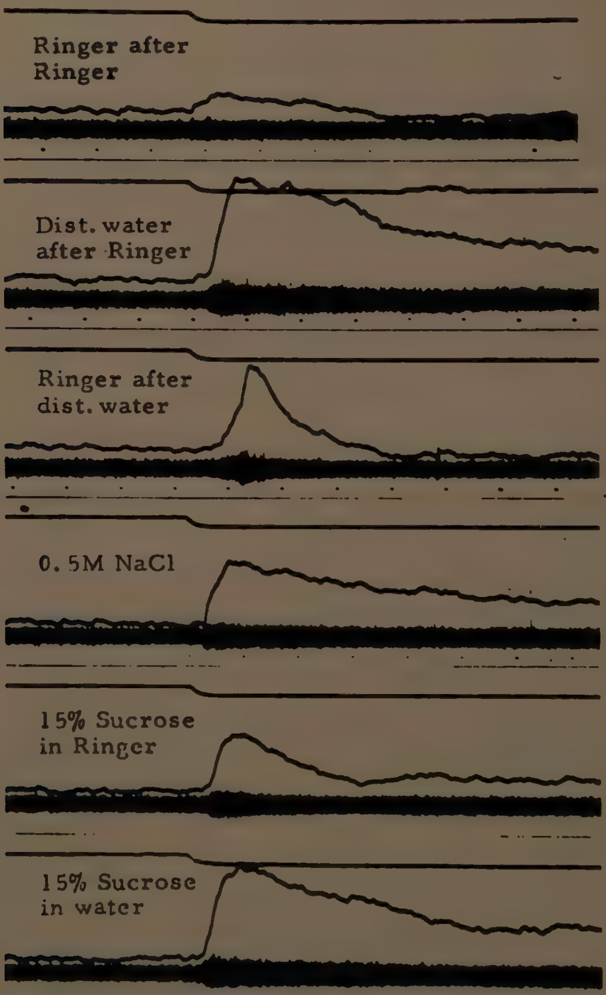

FIGURE 1. Integrated responses (middle tracings) from the intact chorda tympani of the rhesus monkey to the application of various sapid solutions on the tongue. Upper tracings signal the moment of application; lower tracings, the direct response. Time: 1 per second.

concentrations of saccharin seemed to stimulate the bitter-taste fibers (Andersson *et al.*, 1950). Some few-fiber preparations were obtained from the monkey's chorda tympani in which the sweet-taste fibers did not respond to any other sapid solutions except those that in humans taste sweet. An example of this is seen in FIGURE 4, in which the records are obtained from the same nerve strand as in FIGURE 2 after successive subdivisions. In this reduction of the number

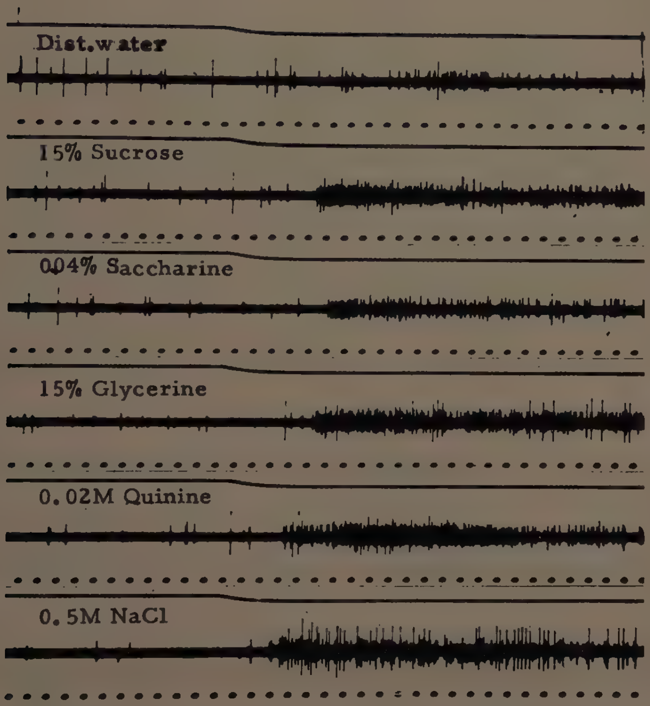

FIGURE 2. Records from a strand of the chorda tympani of the rhesus monkey responding to the application upon the tongue of various substances dissolved in water. Note the large spike response to NaCl. Time: 10 per second.

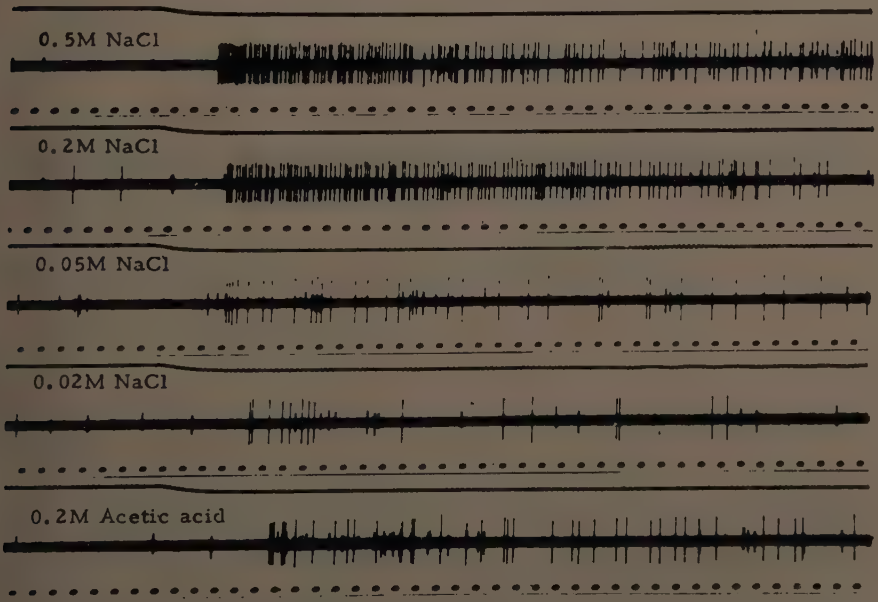

FIGURE 3. Records from a fine strand of the chorda tympani of the rhesus monkey. Note the large spike response to NaCl. Threshold about 0.01 M NaCl. Time: 10 per second.

of fibers we succeeded in getting rid of the fibers that in the original preparation (see FIGURE 2) responded to salt, quinine, and water.

The response to acetic acid obviously derived from a specific acid-taste fiber (see bottom record of FIGURE 4). This fiber produced much bigger spikes than did the sweet-taste fibers in this nerve preparation and did not respond to any other sapid stimulus. Thus we can conclude that the rhesus monkey has specific fibers for salt, sweet, bitter, and acid solutions as well as for water in itself.

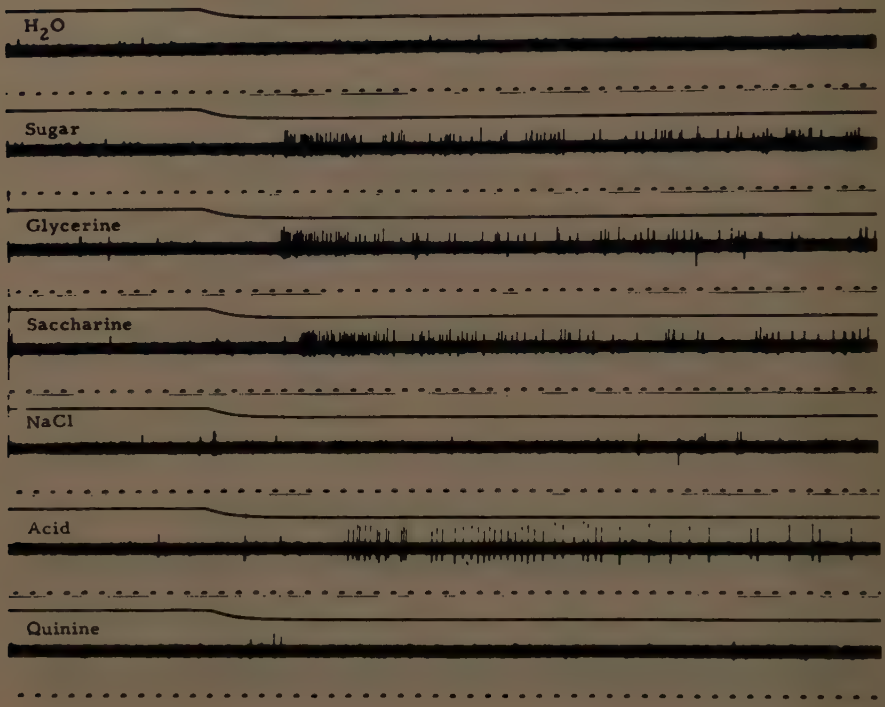

FIGURE 4. Action potentials from the chorda tympani of the rhesus monkey, showing response to different solutions applied to the tongue. Upper trace signals opening of stopcock Time: 10 per second.

Experiments on Humans

A few years ago C. Åhlander and I made our first attempts to place leads on the chorda tympani of man during operation on the middle ear, in the Ear, Nose and Throat Department of the Southern Hospital, Stockholm. Of 10 trials in which the surgeon applied the electrodes to the exposed chorda tympani in the cavum tympani we obtained very weak signals in only 2 cases in response to cold and gustatory stimulation of the tongue. The responses, however, were only just audible in the loud-speaker and could not be recorded.

In recent attempts, made in August and September 1958 in the ear clinic of Karolinska Hospital, Stockholm, in cooperation with H. Diamant, we were able to record the response from the chorda tympani during operations performed in order to mobilize the stapes in the middle ear. In 2 of 6 attempts we have been able to record the integrated electric response of the nerve when the tongue was stimulated by touch and various sapid solutions. As may be seen from FIGURE 5, there was a good response to 0.5 M NaCl solutions and also to 15 per cent sucrose, 0.04 per cent saccharin, 0.02 M quinine sulfate, and 0.2 M acetic acid. The application of water to the tongue, however, was followed by a reduction of the spontaneous activity in the nerve in exactly the same fashion

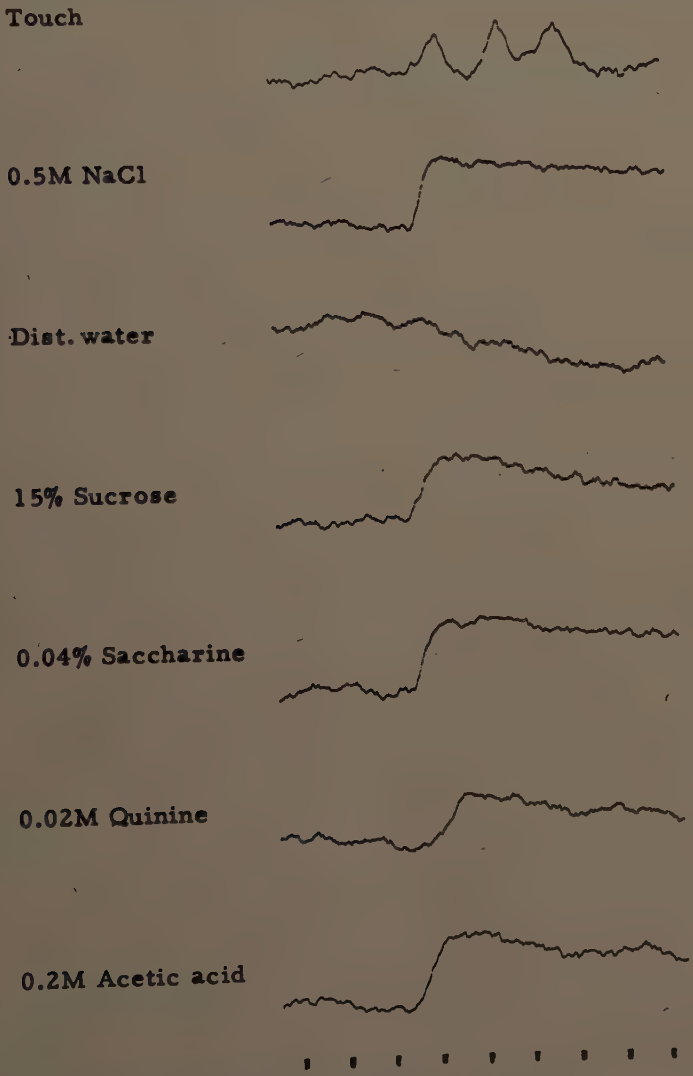

FIGURE 5. Integrated responses of the whole chorda tympani nerve of man to various solutions flowed over the tongue. Time: 1 per second.

that we had previously found in the rat, which does not possess any taste fibers responding positively to water. Thus, man seems to lack a specific water taste, in contrast to the cat, the dog, the pig, and as high a mammal as the rhesus monkey. As our evidence, however, is based upon successful experiments in only 2 humans, we must collect more evidence before being able to say definitely that man lacks a specific water taste. The evidence thus far obtained is that 2 persons definitely lack water taste. Future experiments will tell us whether this holds for all humans or whether, in addition to great species differences, there also exist individual differences in the water taste. Speaking for myself, however, I am inclined to doubt that water elicits any positive taste sensation. The action of water on our gustatory receptors, particularly on the salt-responding fibers, must be of a negative nature in that water decreases or abolishes the resting activity of our taste fibers. A high sensitivity of the salt-taste fibers that can be contrasted with the negative effect of water would thus be the instrument for our discrimination between dilute salt solutions and pure water. It seems to us rather strange that we should lack specific water-responding fibers whereas our next relatives, the monkeys, have such fibers. This fact does not make it easier to understand what physiological purpose the water-taste fibers may serve.

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THE GENERATION OF ELECTRIC ACTIVITY IN A NERVE ENDING*

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When a Pacinian corpuscle is pressed upon, a series of electric events takes place inside the sense organ. The sequence of events may be conveniently summarized by the following scheme:

$$\begin{array}{c}\text{mechanical}\\ \text{stimulus}\end{array} \rightarrow \begin{array}{c}\text{generator}\\ \text{potential}\end{array} \rightarrow \begin{array}{c}\text{all-or-nothing}\\ \text{potential}\end{array} \rightarrow \begin{array}{c}\text{propagated all-or-nothing}\\ \text{potential}\end{array}$$

The generator potential is the earliest detectable sign in the receptor; it succeeds the mechanical stimulus at a fixed latency. The generator potential of Pacinian corpuscles was first described by Álvarez-Buylla and Ramírez de Arellano (1953), and by Gray and Sato (1953). The most salient characteristic of the generator potential is its finely graded nature: its amplitude increases and decreases without detectable steps as a function of stimulus strength. When the generator potential reaches a certain critical amplitude, an all-or-nothing potential is produced inside the corpuscle which, under certain conditions, may propagate along the afferent nerve fiber of the sense organ (Loewenstein and Altamirano-Orrego, 1958*b*).

The present paper deals with two questions. The first refers to the sites at which the electric activity originates in the sense organ. It will be shown that the generator potential and the all-or-nothing potential arise at well-defined and clearly separated areas, namely, at the nonmyelinated ending and at the first intracorpuscular node of Ranvier, respectively. The second question deals with some of the mechanisms by which a mechanical stimulus is converted into electric activity. Evidence will be given that the mechanoreceptive process at the ending is of discontinuous nature, that is, there are functionally independent receptor spots scattered all over the ending; stepless gradation of generator potentials can be brought about by spatial summation of independently active receptor spots.

THE SITES FOR MECHANOELECTRIC CONVERSION

Mechanoreception in Absence of the Adventitious Structure

In a large number of sense organs of the corpuscle type, such as the Grandry, Meissner, Golgi-Mazzoni, Herbst, and Pacinian corpuscles, the sensory nerve ending is surrounded by a relatively well-organized adventitious structure. The mass of adventitious structure is far greater than that of the nervous tissue of the sense organ. This is particularly true of the Pacinian corpuscle in which the ratio of nerve to adventitious tissue volume is about 1:10,000. The question of what is the function of the adventitious tissue goes back to the early days of sensory physiology. Many kinds of functions have been attributed to the adventitious structure. Among other things, it has been regarded as an amplifier of stimuli, as a supporting and protecting element of the nerve ending and, often, as responsible for "receptor specificity." The principal points at issue may be summarized by the following question: does the adventitious structure play an active part in mechanoreception, namely, in the chain of events that transduces stimuli into nerve impulses, or is its action merely a passive one? The Pacinian corpuscle provides a very suitable preparation for a direct approach to this question. Its adventitious structure is large (of the order of 0.1 mg.); it is therefore possible to dissect away selected portions, or almost all of the adventitious structure while, at the same time, the ability of the sense organ to produce generator potentials in response to mechanical stimuli can be tested.

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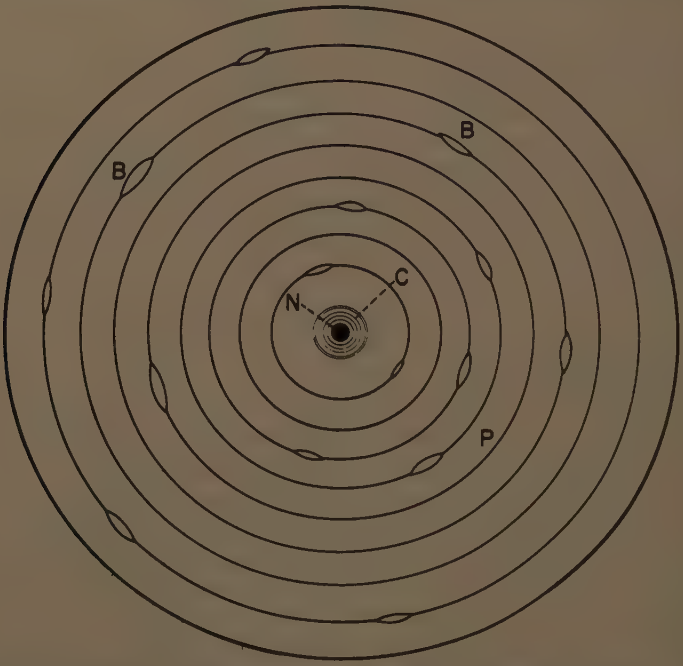

FIGURE 1. Diagram of a transverse section through the central region of a corpuscle (drawn according to electron micrograph data of Pease and Quilliam, 1957). *N*, nonmyelinated nerve ending *C*, inner core; *P*, peripheral zone of the capsule; and *B*, cell bodies of presumable mother cells of lamellae. The thickness of the inner core has been exaggerated. From Loewenstein and Rathkamp, 1958; reproduced by permission from *The Journal of General Physiology.*

Some aspects of the anatomy of a Pacinian corpuscle, as revealed by Pease and Quilliam's (1957) electron micrographs, are illustrated in FIGURE 1. The drawing shows a transverse section across the nonmyelinated nerve ending and the surrounding adventitious structure. The latter is comprised of two structurally different parts: the peripheral zone and the inner core. The peripheral zone amounts to more than 99.99 per cent of the corpuscle's mass. It is made up principally of loosely packed lamellae arranged in a circular fashion. The inner core is a thin sheath of about 10 μ thickness, consisting of compact bilaterally arranged lamellae, which encloses the nonmyelinated ending of the afferent axon. The myelinated part of intracorpuscular axon lies outside the core surrounded by the peripheral zone; there are 1 or 2 Ranvier nodes inside the corpuscle before the axon emerges from the sense organ (Quilliam and Sato, 1955).

All of the experiments described in this paper were performed on isolated Pacinian corpuscles of the mesentery of the cat. The corpuscles were set up in a chamber containing an oxygenated Krebs' solution covered with mineral oil (FIGURE 2). Mechanical stimulation of the receptor was provided by the damped deflections of an electrically driven piezo-electric crystal. A glass stylus (S) attached to the crystal transmitted the deflections to the corpuscle. Brief pulselike deflections of 1-msec. duration, controlled photoelectrically and continuously variable between 0 and 45 μ, were available for stimulation. Unless stated otherwise, the electric activity of the receptor was led off with a fluid electrode, at the point where the axon emerges from the corpuscle, and a platinum electrode (E_1). Under these conditions a generator potential produced at the nonmyelinated ending must spread over about 450 μ of intracorpuscular myelinated axon before reaching the recording electrode. For a detailed description of the set-up and the procedures for stimulation, re-

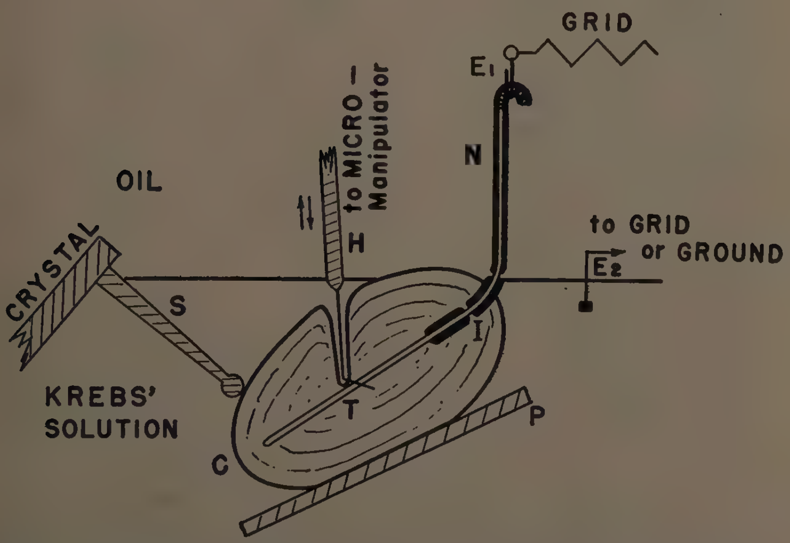

FIGURE 2. Set-up used in the experiments of microdissection and compression. C, corpuscle; T, nonmyelinated nerve ending; and I, first Ranvier node of sensory axon (N). Myelin drawn in heavy black; only the first two nodes are shown. S, glass stylus that transmits deflections of the piezoelectric crystal to the corpuscle; H, steel hook for compression of ending or node. From Loewenstein and Rathkamp, 1958; reproduced by permission from *The Journal of General Physiology.*

cording, and microdissection, see Loewenstein and Altamirano-Orrego, 1958*b*; and Loewenstein and Rathkamp, 1958.

FIGURE 3 illustrates an experiment in which the lamellae of a living corpuscle have been progressively peeled off. Two stages of dissection (*b* and *c*) are shown; below each are the electric responses to mechanical stimuli corresponding to each stage. In the final stage practically all of the peripheral zone has been removed, leaving only the inner core intact. It is seen that, in spite of the removal of more than 99.99 per cent of the original mass of the corpuscle,

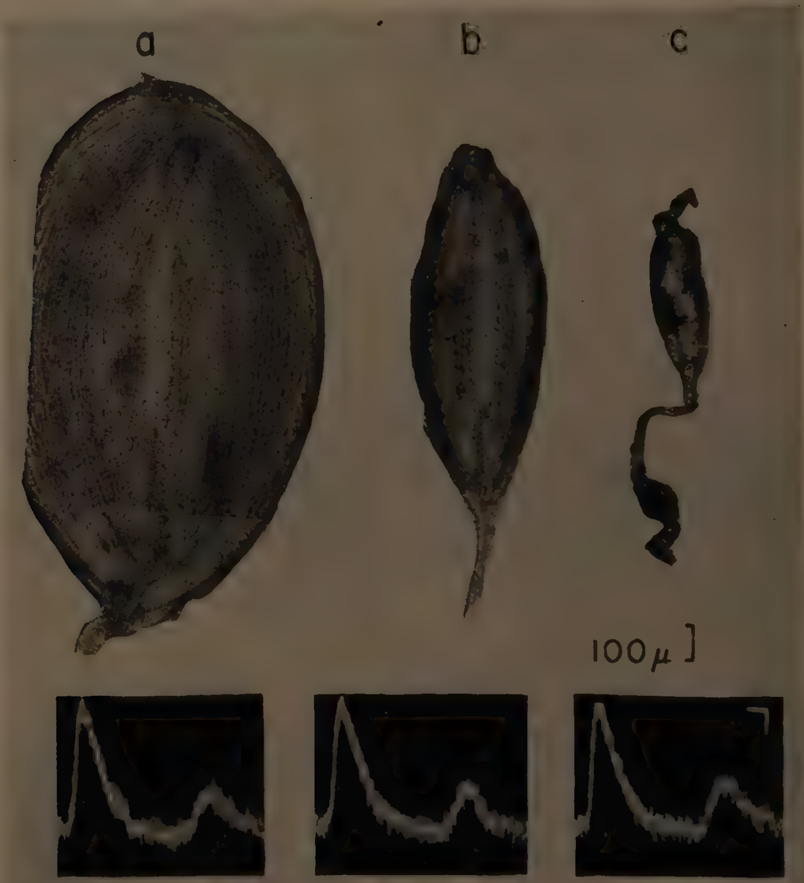

FIGURE 3. Mechanoresponsiveness of a sense organ after removal of adventitious structure. Phase contrast photomicrograph of three stages of dissection of a living unstained corpuscle; *a*, corpuscle intact; *b* and *c*, the same corpuscle after elimination of the peripheral zone. In *c* only the inner core and its enclosed nerve ending and axon remains intact. The corresponding responses are shown below each stage of dissection. Two mechanical stimuli are applied successively. The first produces an all-or-nothing potential; the second (subthreshold) causes only a generator potential. Note that mechanoelectric conversion remains unimpaired, in spite of the removal in *a* of more than 99.99 per cent of the sense organ's structure. Calibration, 1 msec.; 25 μV. From Loewenstein and Rathkamp, 1958; reproduced by permission from *The Journal of General Physiology.*

the mechanoreceptive ability of the preparation remains unimpaired. The preparation, consisting of a nerve ending, a piece of axon, both surrounded by the thin inner core, is essentially as good a mechanoreceptor as is the intact corpuscle: it produces generator potentials in response to mechanical stimuli of the same order of magnitude as does the intact organ. It is clear from these results that the peripheral zone is not required for mechanoelectric conversion.

Integrity of the small remaining fraction of adventitious structure is also not required for mechanoelectric conversion. The inner core can be punctured all over with a fine needle, or multiple incisions can be made into it without causing the preparation to lose its characteristics as a mechanoreceptor, provided the nerve ending is not injured. Because of intimate connections between the inner core and the nerve ending, the former cannot be entirely removed. However, its outer layers can be stripped off and, in addition, small pieces of inner-core tissue can be excised without causing any essential change in the ability of the preparation to convert mechanical stimuli into generator potentials (Figure 4).

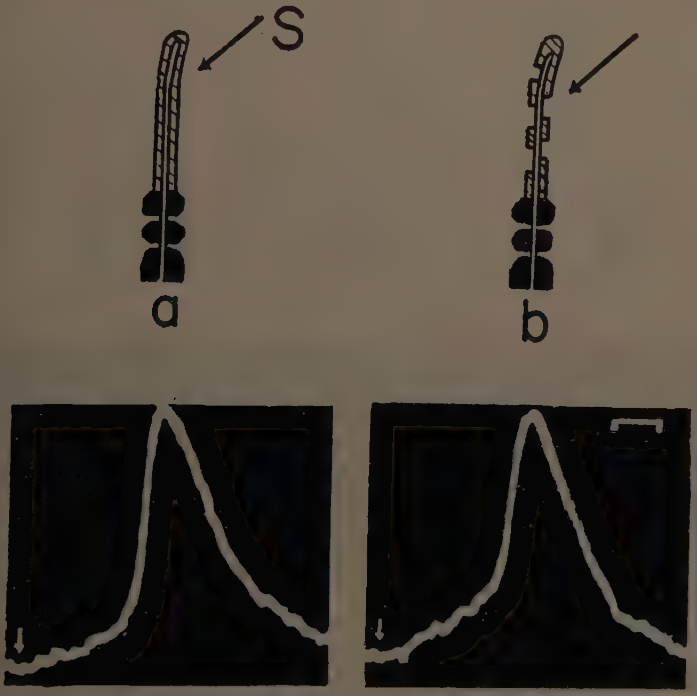

Figure 4. Mechanoresponsiveness after partial destruction of inner core. A decapsulated preparation, such as shown in Figure 3*c*, consisting of a nerve ending, axon, and inner core, is stimulated mechanically, eliciting an all-or-nothing response on top of a distinct generator potential: *a*, before; *b*, after resection of multiple pieces of inner core. Calibration, 1 msec. From Loewenstein and Rathkamp, 1958; reproduced by permission from *The Journal of General Physiology.*

These results show that the structures of the peripheral zone do not participate in mechanoelectric conversion, and that the receptor can dispense with at least a large part of its inner-core structure without impairment of mechanoelectric conversion.

Origin of the All-or-None Potential

The decapsulated corpuscle the peripheral zone of which has been removed provides a convenient preparation for a study of the origin of the generator and the all-or-nothing potentials. It has a long and straight nonmyelinated ending, and the adjacent first Ranvier node, which normally lies inside the corpuscle, becomes exposed after removal of the capsule. Both ending and node can be viewed in the living preparation under a phase-contrast or dark-field microscope. The greatest advantage of the decapsulated preparation lies in the accessibility of the ending and axon for compression. Thus it is possible to block selectively the activity of certain parts of intracorpuscular nerve and axon by applying pressure to them with a fine steel hook. If the first Ranvier node is compressed, all sign of all-or-nothing activity disappears inside the sense organ, and finely graded generator potentials then constitute the only remaining response to mechanical stimulation (FIGURE 5). The compression of any other region of the myelinated axon, even as near as 10 μ to the first node, does not abolish the all-or-nothing potential. This result reveals that the first intracorpuscular Ranvier node is the site of origin of the all-or-nothing potential inside the sense organ.

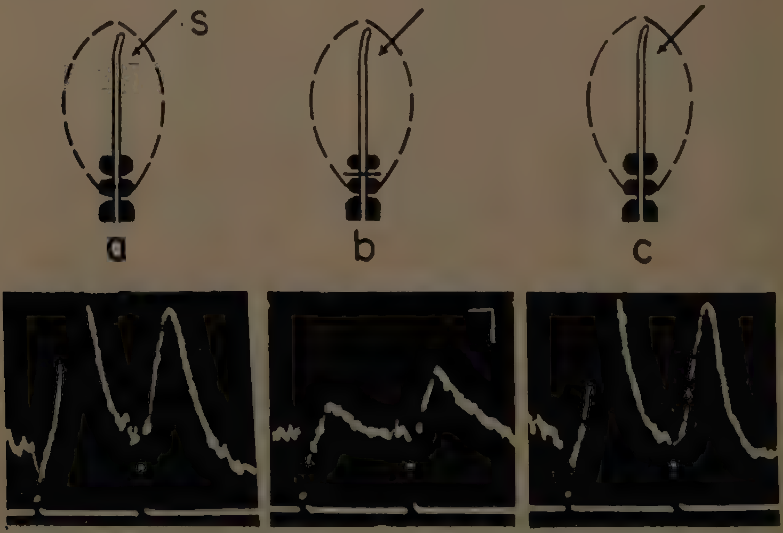

FIGURE 5. Abolition of all-or-nothing potentials upon compression of Ranvier node. At *a*, 2 successive mechanical stimuli are applied to the corpuscle, producing 2 all-or-nothing potentials. The lower beam signals the relative magnitude of mechanical stimuli. At *b*, the same stimuli as before are again applied, while the first intracorpuscular Ranvier node is being compressed, now eliciting only 2 generator potentials. At *c*, reversal of effect after pressure from node is released. Calibration, 1 msec.; 25 μV. From Loewenstein and Rathkamp, 1958; reproduced by permission from *The Journal of General Physiology.*

After the preceding results the nonmyelinated ending was left as the only possible site for the production of the generator potential. In fact, if the ending is compressed along its entire length, all sign of generator potentials in response to mechanical stimuli disappears, and the preparation, of course, is thus rendered incapable of producing nerve impulses (see *f*, FIGURE 10, below).

Further evidence that the generator and all-or-nothing potential are set up at different membrane sites comes from experiments in which the refractory states of the two potentials were studied (Loewenstein and Altamirano-Orrego, 1957, 1958*a*, 1958*b*). Both the generator and all-or-nothing potential leave a refractory condition behind, but the natures and durations of their respective refractory states are quite different; for example, whereas the all-or-nothing potential has an absolute refractory period, the generator potential has no absolute refractory period; instead, it decreases continuously in amplitude as the interval between conditioning and generator potential is progressively shortened. During the relative refractory period of the all-or-nothing potential the critical firing level increases exponentially (FIGURE 6). It is possible, therefore, to produce large generator potentials (say, 80 per cent of an all-or-nothing potential) during the early part of the relative refractory period left by a preceding all-or-nothing potential without firing of all-or-nothing potentials (FIGURE 7). This is another instance in which blockage of the all-or-nothing potential, this time caused by rendering the node refractory rather than pressing on it, causes the receptor to produce only finely graded generator potentials when mechanically stimulated. That the generator and all-or-nothing potential may have

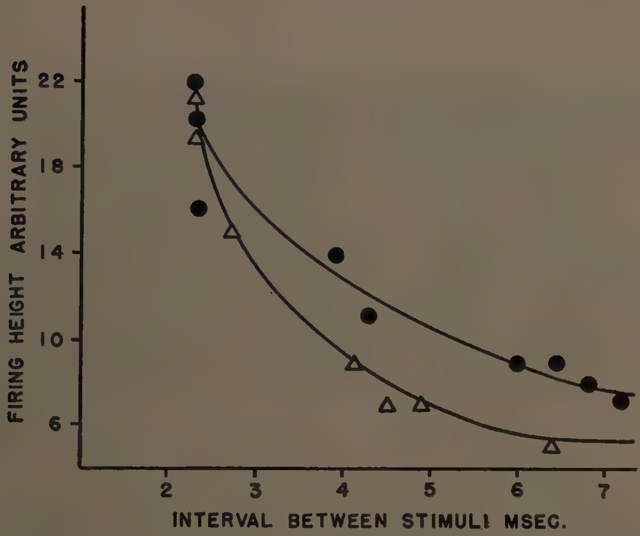

FIGURE 6. The critical firing level during the refractory period of the all-or-nothing potential. The firing height, namely, the minimal generator potential which produces an all-or-nothing potential, is plotted against the interval between two successive mechanical stimuli. The symbols ● and △, data from two different corpuscles. From Loewenstein and Altamirano-Orrego, 1958*b*; reproduced by permission from *The Journal of General Physiology.*

independent origins is furthermore suggested by the findings of Diamond *et al.* (1956), who separated the two potentials by anelectrotonus.

An observation of particular relevance to the question of the origin of the generator and all-or-nothing potential was made when the mutual influence of the refractory states of the two potentials was studied (Loewenstein and Altamirano-Orrego, 1957, 1958*a*, 1958*b*). It was then found that, although both the generator potential and the all-or-nothing potential each leave a refractory state behind, there is no mutual influence between the refractory states of the two potentials. Thus, a generator potential falling on the refractory trail left by a preceding generator potential is reduced, but it is not affected when it falls on the refractory trail left by a preceding all-or-nothing potential (FIGURE 8). The result is explained by the fact that the generator potential arises at the membrane of the nonmyelinated ending, while the all-or-nothing potential is set up at the first Ranvier node, which lies at an average distance of 250 μ from the ending.

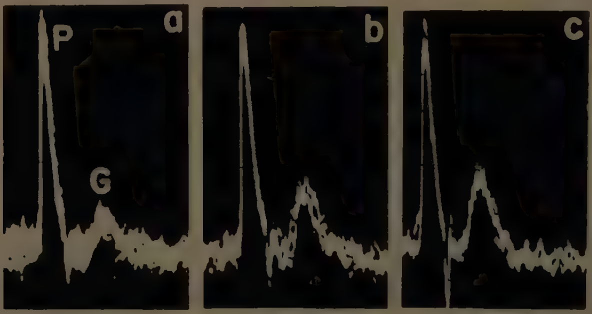

FIGURE 7. A pure generator potential is produced during the refractory period of the all-or-nothing potential. Two successive mechanical stimuli are applied at a constant interval. The first stimulus is held at constant strength producing an all-or-nothing potential (*P*). The second, falling within the refractory period of *P*, produces a graded generator potential (*G*). The strength of second stimulus increases from *a* to *c*, producing a proportionally increasing generator potential. From Loewenstein and Altamirano-Orrego, 1958*b*; reproduced by permission from *The Journal of General Physiology.*

PROPERTIES OF THE NONMYELINATED ENDING

Absence of Regenerative Potentials

If the node is blocked by pressure or by refractoriness, the only responses of the nonmyelinated ending to mechanical stimuli, however strong they are, are finely graded generator potentials (see FIGURE 5). This reveals that the nonmyelinated nerve ending is incapable of producing regenerative potentials when stimulated mechanically. The question then arises whether this behavior of the ending, since it is observed with mechanical stimulation, is due to some peculiarity of the early transducer stages prior to the generator potential, or whether the membrane of the ending, as opposed to that of the adjacent node, lacks regenerative mechanisms. A direct approach to the question is to pass an outward-flowing current across the ending and to see if and how its membrane may be excited. I have tried to excite the ending electrically with a pair of microelectrodes placed on its surface, but I have not been able to produce a response that may be attributed to the ending alone. However, in order to prevent effective spread of current to regions other than the ending, it was necessary to limit the currents to 10^{-8} amp.

Clear results were obtained by another approach. The attempt was made to excite the ending with the action current of the adjacent Ranvier node; FIGURE 9 illustrates an experiment. A pair of microelectrodes is placed in contact with the nonmyelinated ending. The stylus of the stimulating crystal delivers a small mechanical stimulus to a region of ending close to one of the electrodes (E_1). This causes depolarization of an area of membrane below the electrode; that is, it elicits a rather sharply localized generator potential of subthreshold amplitude (FIGURE 9). Essentially the same results are obtained by moving the stimulating stylus to another site on the ending and placing an electrode in its vicinity. The sensory axon outside the corpuscle then is stimulated with a pair of electrodes placed on the axon at a distance of about 5 cm. from the corpuscle. This produces an impulse that travels antidromically into the sense organ. The impulse can be traced as an all-or-nothing potential as far as the first Ranvier node (FIGURE 9*b*), but no active response is detected from the membrane below the electrodes at the ending (FIGURE 9*b*) nor in any other place of the ending when its surface is scanned with an electrode. This reveals that an antidromic impulse does not excite the nonmyelinated ending.

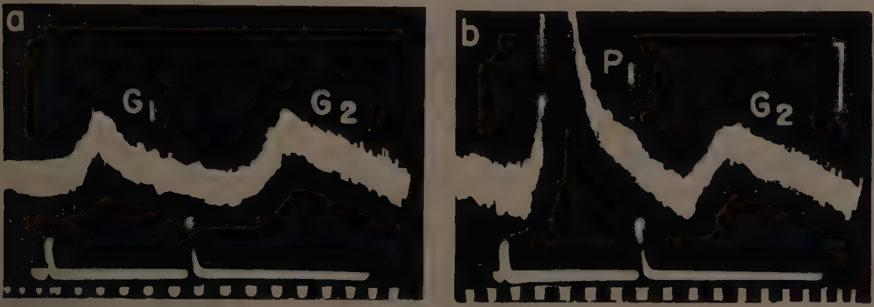

FIGURE 8. The generator potential is not affected by the refractory state of an all-or-nothing potential. Pairs of mechanical stimuli are applied. The first stimulus is critically at threshold, producing either *a*, a generator potential (G_1), or *b*, an all-or-nothing potential (P). The second stimulus succeeds the first at a constant delay. Note that the resulting generator potential (G_2) is essentially of the same amplitude whether the preceding event is a generator or a several-times-greater all-or-nothing potential. If, instead of an all-or-nothing potential (P), the response to the first stimulus in *b* had been a generator potential as large as P_2, the refractory state from such a generator potential would have reduced G_2 below noise level. Calibration, 1 msec. between dots; 25 μV. From Loewenstein and Altamirano-Orrego, 1958*b*; reproduced by permission from *The Journal of General Physiology.*

For the recording of the action current from the first node, the nerve fiber and ending were pulled into an insulator device with two partitions (FIGURE 9), similar to that employed by Huxley and Stämpfli (1949) for frog axons, and some portions of nonmyelinated ending were previously exposed by excising pieces of inner core (see FIGURE 4).

The action current of the node is at least 5 times greater than the minimal current required for dromical excitation of the node, since a mechanically induced generator potential of one fifth the amplitude of a normal action potential satisfies amply the firing threshold of the node. Consequently, when an antidromic impulse enters the corpuscle, a current at least 5 times stronger than that required for nodal excitation flows between node and ending; yet the ending is not excited by it. The current density at the ending may be expected to be much lower than at the node because of the much larger nonmyelinated area at the ending. Assuming a uniform distribution of current inside the core, a rough estimate of current density relations may be made on the basis of the areas of node and ending. It is then obtained that the current density at the node is about 240 times that at the ending. An estimate of this kind may be off the true value by a considerable factor if there are foldings in the nodal membrane. Nevertheless, the difference in current density, even if the density

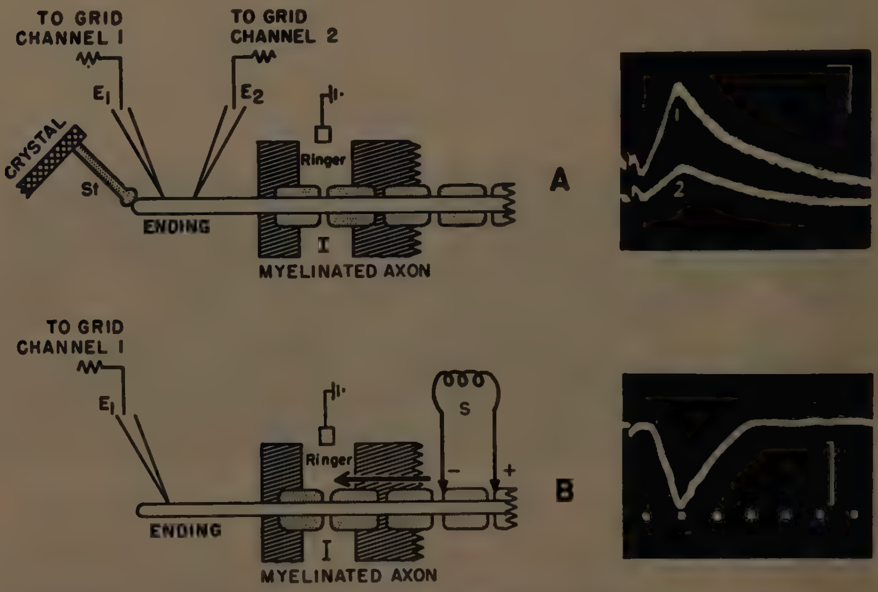

FIGURE 9. (*A*) Local activity confined to a small membrane portion of ending. Two microelectrodes connected to separate amplifier channels are placed about 350 μ apart in contact with the nonmyelinated ending of a decapsulated corpuscle. E_1 is placed at a distance of approximately 20 μ from the stimulating stylus (*St*). The first Ranvier node (I) is grounded and insulated from the ending. The upward deflection of the beam means "ending negative." Beams 1 and 2 give the potentials in response to a single mechanical stimulus, as recorded by electrodes E_1 and E_2, respectively. Note the smaller amplitude and slower rate of rise of the potential of the more distant electrode E_2. Calibration, 50 μV; 1 msec. (*B*) An antidromic impulse does not excite the nonmyelinated ending. Same corpuscle as in *A*. The axon outside the corpuscle is now stimulated electrically, causing an antidromic impulse to travel into the corpuscle. The impulse can be traced as an all-or-nothing potential as far as the first node, but no active response is detected at the ending. Calibration, 200 μV; 1 msec.

at the node were only 24 times that of the ending, may explain why the ending is not excited by antidromic stimulation. However, it still remains a remarkable fact that the ending cannot be invaded by an antidromic impulse, and the related question why the mechanically activated ending is incapable of giving a regenerative response still calls for an answer. For the latter question, the problem of current densities plays no role since, as shown below, active receptor sites lie close to each other all over the membrane of the ending. The principal difference between the ending and a structure with an all-or-nothing behavior, such as the axon, lies probably in their mechanism of excitation. At the axon, activation of the membrane is caused by electric current; at the ending, however, activation is produced by deformation of the membrane, but almost certainly not by electric current. A generally recognized condition for the production of regenerative potentials is the ability of a membrane portion to be excited by local currents from an adjacent membrane portion. However, as was seen in the preceding experiments, the membrane of the ending has a high threshold, if at all, for electric excitation. It is conceivable, therefore, that the current from a portion of membrane which has been activated by deformation does not excite an adjacent undeformed portion. Electric activity may thus be restricted to that membrane part which has been deformed, instead of spreading over the entire length of ending in a decrementless manner.

Another kind of experiment of quite different nature also indicates that the ending is not excited by the action current of the adjacent node. It has recently been found that the generator potential of a Pacinian corpuscle becomes enhanced when the ending has been previously submitted to mechanical stimulation of high frequency (Loewenstein and Cohen, 1958*a*). This kind of post-tetanic potentiation of the potential of a single nerve ending is a rather large effect; the generator potential is often found to increase its original size by 50 per cent. The effect can be produced, however, only by dromic tetanic activation of the ending, namely, by repetitive mechanical stimulation of the receptor, but not by impulses fired antidromically into the sense organ at the same frequency as the dromic stimuli.

The experiments on the refractory state of generator and all-or-nothing potential, already considered in FIGURE 8, provide further evidence along this line. The finding that the refractory states of the all-or-nothing and generator potentials are mutually independent reveals again that activity at the node does not excite the membrane of the nonmyelinated ending.

In summary, two properties of the nonmyelinated ending now appear well established: (1) it does not produce all-or-nothing potentials when mechanically stimulated, and (2) it is not excited by an antidromic impulse.

Because of the first property, the nerve ending of Pacinian corpuscles may be classified with certain other electrogenic tissues, such as the motor end plate of skeletal musculature (compare Castillo and Katz, 1956) and the dendrites of cortical (Purpura and Grundfest, 1956) and other nerve cells (see Grundfest, 1957, for a detailed analysis of this question) which appear to be incapable of producing regenerative responses. Of particular relevance to the present case are the dendrites of the stretch receptor nerve cell of crustaceans. These dendrites produce finely graded generator potentials, but there is no evidence for production of all-or-nothing potentials in them (Eyzaguirre and Kuffler, 1955); the all-or-nothing potential arises at the axon, at some distance from the dendrites and the soma of the nerve cell (C. Edwards and D. Ottoson, personal communication).

The second property confers on the sense organ a valve system that permits a one-way traffic only at its terminal portion. This unidirectional system exists without having any visible structural solution of continuity such as that of a typical synapse. There is at least one valve at the stage of conversion of mechanical into electric energy, and another one between ending and node, both viable only in the direction of ending to node. This may be of physiological importance in a receptor network in which several receptors are innervated by branches of one single axon. Such networks have been found in frog muscle spindles (Gray, 1957), in various types of skin receptors (Adrian *et al.*, 1931; Maruhashi *et al.*, 1952; Hensel, 1952; also, see Granit, 1955; Wedell *et al.*, 1955; Loewenstein, 1956; Bullock and Diecke, 1956; and Catton, 1958), and in some mesenteric Pacinian corpuscles (Loewenstein and Rathkamp, unpublished data). In the absence of a one-way arrangement, a rather inoperative situation might occur; the impulse originated in one receptor may be fired antidromically into another ending, and there, if the depolarization of the ending were to outlast the refractory period of the node, it would generate reverberating dromic impulses.

Discontinuous Nature of the Transducer

Receptor sites. The experiments hitherto described showed that the mechanoelectric transducer, or at least the final stages of it, are localized at the membrane of the nonmyelinated nerve ending. The question now arises whether the transducer process is continuous or discontinuous. The two following main possibilities may be considered: (1) there is one single transducer connected to and encompassing the entire length of membrane of the nonmyelinated ending; (2) there are multiple transducers scattered all over the ending. Whatever the answer may be, it must account for the properties of the generator potentials, namely, its continuously graded nature, its fixed latency, its "time" and "size" factor of refractoriness, and its spontaneous decay (see Loewenstein, 1958). The results of the following experiments will lend support to the idea that the transducer process at the ending of Pacinian corpuscles is of a discontinuous nature.

In a corpuscle in which most of the capsule has been removed, a selected portion of nerve ending can be compressed with a fine steel hook (FIGURE 10). If, for example, a distal portion of ending is thus compressed, mechanoelectric conversion is abolished only in the compressed part of ending; the remaining intact central stump of ending continues to set up generator potentials when a mechanical stimulus is applied to it (FIGURE 10*a–e*). In a large-sized corpuscle the ending has a length of about 600 μ. This makes it usually possible to repeat several times the above procedure of partial compression. The result is always the same: as long as there is a piece of intact ending left, a generator potential is produced in response to mechanical stimulation. These results reveal that integrity of the nonmyelinated nerve ending is not required for mechanoelectric conversion.

A simple demonstration of subsistence of mechanoreception in a fragment of nerve ending can be given in a corpuscle of which a distal portion containing a piece of nonmyelinated ending has been cut off. It is then found that the central remains of the corpuscle continue to produce generator and eventually also all-or-nothing potentials in the same way as before amputation (FIGURE 11). In the experiments of compression, as well as those of amputation, the intact fragment of ending continues to respond to mechanical stimuli of a similar order of magnitude (crystal deflections of the order of 0.1 μ) as before compression or amputation.

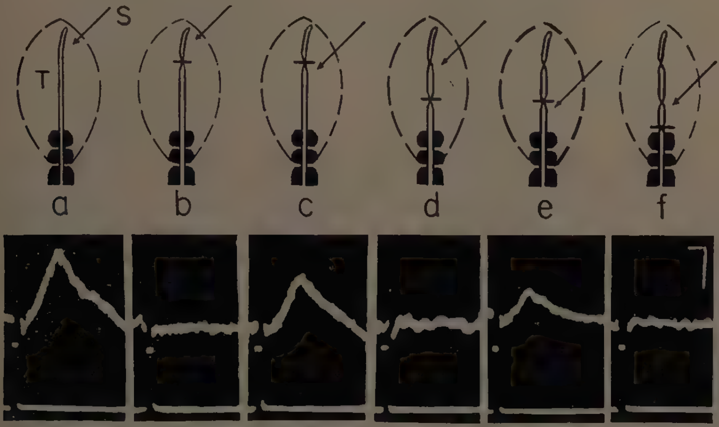

FIGURE 10. Mechanoelectric conversion after partial compression of the nonmyelinated ending. A subthreshold mechanical stimulus of constant strength is applied to the inner core of a decapsulated corpuscle (*a* to *f*); only in *a*, *c*, and *e* is a generator response detected. Lower beam signals relative magnitude and duration of mechanical stimuli; upper beam, the electric activity of the ending led off the myelinated axon (see text). The arrows of the diagram indicate the zone of application of mechanical stimuli. The horizontal lines across the nerve ending (*T*) indicate the central boundary of compressed area of ending. At *a*, generator response of the intact ending; at *b*, after compression of a distal portion of ending; at *c*, after moving stimulus application point beyond the compressed zone; at *d*, after compressing a zone located centrally with respect to stimulus application point; and at *f*, after compression of entire length of ending. Calibration, 1 msec.; 25 μV. From Loewenstein and Rathkamp, 1958; reproduced by permission from *The Journal of General Physiology*.

The above results suggest that the transducer is of discontinuous nature; they may be interpreted to mean that there are functionally independent transducer sites distributed all over the ending. The results thus far, however, are not necessarily incompatible with the possibility of a normally continuous transducer structure, fragments of which still retain transducer properties.

Spatial summation of receptor sites. It would seem convenient, at this point, to examine the principal consequences that would derive in the case of a discontinuous transducer structure. The hypothesis of independent transducer parts was originally postulated to explain certain observations on the refractory generator potential (Loewenstein, 1958). It was then proposed that the generator potential arises at functionally independent transducer sites (generator units) scattered all over the membrane of the nonmyelinated ending. As a first consequence of such a situation, a fragment of nerve ending should be as good a mechanoreceptor as the intact nerve ending. That this is the case is revealed by the experiments described above. As a second consequence, it should be possible to activate independently different transducer sites of the ending; hence, it should be possible to summate generator potentials of dif-

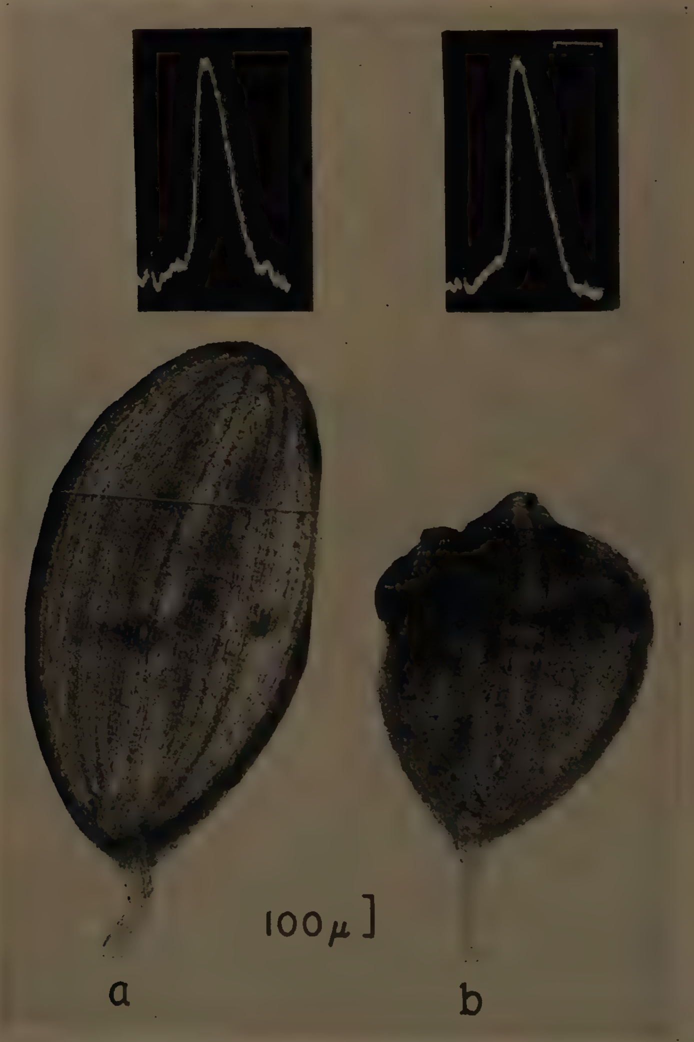

FIGURE 11. Mechanoelectric conversion in a fragment of nerve ending. A suprathreshold mechanical stimulus produces an all-or-nothing potential on top of a generator potential: *a*, in the intact corpuscle; *b*, after cutting off a distal piece of corpuscle that includes a stretch of nonmyelinated ending of about one fourth of the ending's original length. Phase contrast photomicrographs of an unstained preparation. The horizontal line across *a* is due to the composition of two successive photomicrographs; the image of the entire corpuscle was too large to fit into the photographic field. From Loewenstein and Rathkamp, 1958; reproduced by permission from *The Journal of General Physiology.*

ferent transducer sites by spatial summation of stimuli along the ending. The following experiments were done to test the second consequence.

In one type of experiment, two different regions of ending were simultaneously stimulated. The stimulating styli of a pair of independent crystals were each applied to a different region of ending of a decapsulated corpuscle (FIGURE 12). When stylus No. 1 alone delivered a weak single stimulus, the electrodes placed on the myelinated axon near the ending recorded a generator potential of a certain subliminal amplitude (FIGURE 12*a*). When stylus No. 2 alone delivered a stimulus, a generator potential of a similar amplitude as before was recorded (*b*). If both stimuli were delivered simultaneously, summation of the generator potentials occurred (*c*). By delaying the stimulus of stylus No. 2 with respect to that of No. 1, spatial summation could be shown at any interval shorter than the decay time of a single generator potential (*d*). There can be no question of an artifact due to mechanical summation of stimuli or due to spread of stimuli, for if two equal stimuli are delivered successively at an interval of, for example, 1 msec. by stylus No. 1 to the same site, the second stimulus evokes no detectable response because it falls on the refractory trail of the first (*d*). Moreover, direct evidence is given below that spread of stimuli does not occur over any appreciable length of ending under the present experimental conditions.

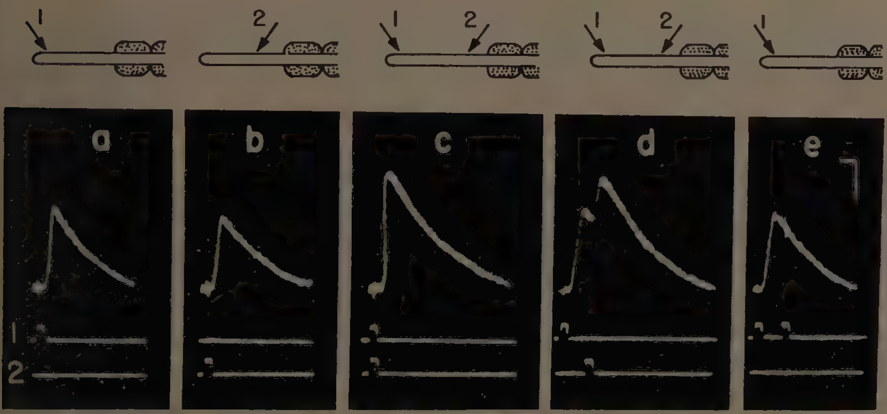

FIGURE 12. Spatial summation of generator potentials in a single nerve ending. Styli 1 and 2 belong to independent stimulating crystals that deliver stimuli to 2 separate regions of ending. Middle and lower beams signal stimuli of styli 1 and 2, respectively. Upper beam for generator potentials as recorded at the myelinated axon. At *a*, stylus 1 alone delivers one stimulus; at *b*, stylus 2 alone delivers one stimulus; at *c*, styli 1 and 2 each deliver one stimulus simultaneously; at *d*, stylus 2 applies a stimulus 1 msec. delayed from that of stylus 1; at *e*, stylus 1 alone delivers two stimuli succeeding each other at a delay of 1 msec. Calibration: 1 msec.; 50 μV.

In another type of experiment, 2 microelectrodes are placed in contact with the decapsulated nerve ending (FIGURE 9*a*). A weak mechanical stimulus is delivered to one part of the ending close to electrode E_1. This causes depolarization of an area of membrane in the proximity of the stimulating stylus. The depolarization is recorded by electrode E_1 as a small "generator potential." Electrode E_2 about 350 μ apart still detects this response, but the peak voltage and the rate of rise of the response are markedly reduced. By systematic scanning of the surface of the ending with an electrode, it is found that the amplitude of the response decreases exponentially with the distance between stylus and electrode. Such a result is to be expected if the depolarization is restricted to an area of ending around the stimulating stylus.

The experiments described above reveal that excitation, as well as the refractory state, is confined to that region of the membrane that has been stimulated mechanically but does not spread to adjacent nonstimulated regions of the same membrane by local-circuit stimulation. The electric activity at the ending appears, therefore, to arise at functionally independent membrane regions, henceforth termed receptor sites. The term site is here used in its widest sense. It does not imply structural discontinuities in the membrane as, for example, in a mosaic structure; it only implies discontinuities in the process of activation. As far as the supporting evidence is concerned, a receptor site has only a functional meaning, namely, that of a membrane region that can be excited independently of others. The notion of receptor sites can be made sufficiently inclusive to be fitted into the widely accepted picture for excitable tissues, namely, that of a semipermeable membrane separating 2 ionic media. A receptor site then becomes a membrane "pore" or group of "pores" that can be activated for charge transfer by means of mechanical stimulation. However, the essential property of the present receptor membrane, which makes it so different from the membrane of the adjacent myelinated axon or that of other structures with regenerative responsiveness, is that its constituent sites can be activated mechanically but not by local current from contiguous mechanically activated sites of the same membrane. This concept is supported by three results reported in this paper: (1) no active response is found at the ending when currents are passed through it; (2) the ending is not excited by the currents of antidromic impulses; and (3) active depolarization is confined to that region of the ending which has been stimulated mechanically.

If, in the experiment shown in FIGURE 9*a*, the stimulus strength is progressively increased, it is found that the area of actively depolarized membrane increases as a function of stimulus strength. Since the experiment shown in FIGURE 12 revealed that the electric activity of the various membrane regions does add up by spatial summation, the spatial summation of the electric activity of active receptor sites emerges from these experiments as a possible mechanism for the stepless increase in generator potential as a function of stimulus strength that is recorded with an electrode placed at the initial portion of myelinated axon. Such an electrode integrates the generator potentials from active receptor sites in much the same way as does the first node of Ranvier under normal conditions in the sense organ. It will be emphasized once more that the existence of independent receptor sites in the Pacinian corpuscle does not necessarily imply structural discontinuity of the ending's membrane, but requires only that each receptor site can be independently activated.

Electric model of the receptor. An attempt can now be made to give a model of the receptor (FIGURE 13). The results so far described reveal that there are functionally independent receptor sites, the electric activity of which may be integrated by spatial summation, but they give no information on the input-output relation at each receptor site. As to the latter question, there are two possibilities: (1) the electric output of each receptor site may be all-or-nothing with respect to strength of mechanical stimuli, or (2) the output of each site may be graded with respect to stimulus strength. The resolution obtained with microelectrodes does not allow a decision between the two possibilities. The chief objective of the model is, therefore, to determine whether summation of activity of receptor sites with an all-or-nothing output may conceivably account for the observed stimulus strength/generator potential function.

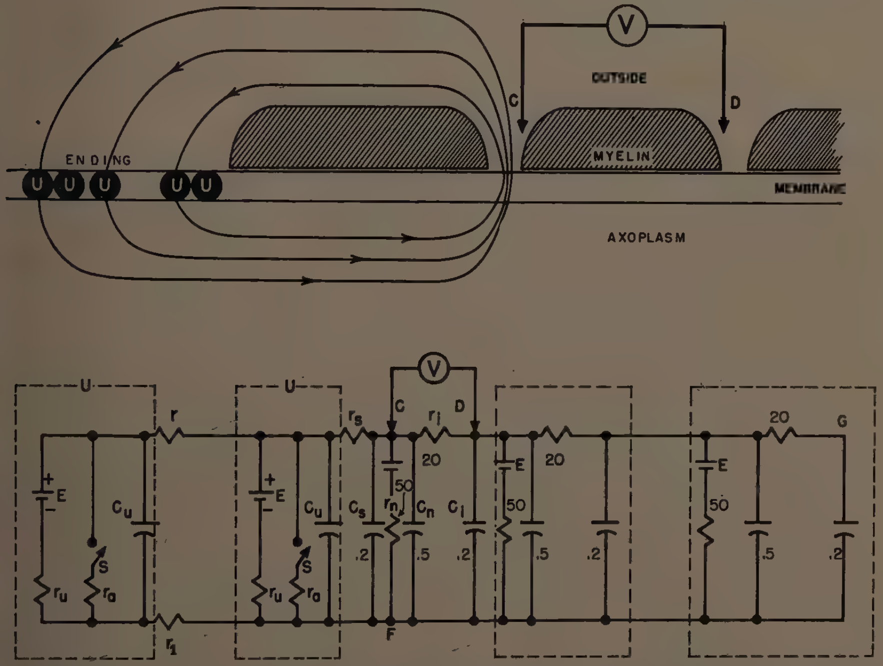

FIGURE 13. Electric model of a receptor. Resistance values in megohms; capacitance in $\mu\mu$farads. The transmembrane potential (*E*) across a receptor site (*U*) is assumed to be 100 mV at rest; it is assumed to drop to a fraction of this voltage when the unit is stimulated mechanically. See text for notations and a detailed description. The upper diagram represents some morphological aspects of the receptor. *U* represents one of many receptor sites assumed to be distributed uniformly over the membrane. These sites do not necessarily imply discontinuity in the membrane structure; they imply only the existence of multiple receptive regions that can be activated independently of each other. Current is represented as flowing between active receptor sites of the ending and the first Ranvier node *C*. The potential difference between the first (*C*) and second node (*D*) is calculated for the equivalent points of the analogue.

Determinations of electric constants of the fiber and ending have not been made in Pacinian corpuscles. Resistance and capacitance values for the "myelinated portion" of the model have been taken from studies of Huxley and Stämpfli (1949) and Tasaki (1952) on myelinated fibers of the frog (see also Stämpfli, 1954), adjusting for the much shorter internodal length (250 μ) of the fiber of Pacinian corpuscles (Quilliam and Sato, 1955). Although it is not likely that the assumed values will differ substantially from the true ones, exactness of these values is not required for a general solution of the problem. The following assumptions have been made: (1) the transverse resistance and capacitance of the membrane of the nonmyelinated ending per unit area is similar to that of a Ranvier node; (2) resistance and capacitance are uniformly distributed over the membrane of the ending. The outside resistance of the ending (r) and the resistance of the axoplasm (r_1) have been neglected. The resistance between ending and node is represented by r_s, and the corresponding capacitance by C_s. Each node and internode is represented by the following elements:

r_n = transverse nodal resistance
C_n = nodal capacitance
r_i = resistance of an internode

Twenty identical nodal sections submerged in mineral oil, two of which are shown in the diagram of FIGURE 13, are considered for the model. The voltage change (henceforth called response) resulting from the activation of receptor sites is measured between the first two nodes of Ranvier (C and D).

In order to assign capacitance and resistance values to a restricted active portion of membrane, the membrane of the ending will be subdivided into m equal parts, each part containing one receptor site. Such a membrane part will henceforth be called a receptor spot. It should be noted that a receptor spot is merely a consequence of the above assumption that capacitance and resistance are uniformly distributed over the membrane of the ending, but has no real existence as in a mosaic structure. Each receptor spot is represented by the elements contained in rectangle U. An inactive receptor spot consists of a voltage source E, representing the transmembrane potential; r_u, representing the transverse membrane resistance at rest; and C_u, the capacitance of the spot. When a receptor site is activated by a mechanical stimulus, it is assumed that E and the transverse membrane resistance of the spot drop to a fraction of their original value. This is represented by switching resistor r_a into the circuit as shown in FIGURE 13. For the derivation of EQUATION 1, r_a is considered to be one-ninth of r_u. Thus, when r_a is connected, the effective value of E drops to 10 per cent of its original value, and the total transverse membrane resistance of the spot also drops to 10 per cent of its original value. This 10 per cent drop was chosen arbitrarily in order to obtain a numerical solution. If the resistance were to drop by a smaller fraction, which at present appears just as likely, the peak response would be smaller (see EQUATION 1); otherwise the essential conclusions of the analysis would be the same. A total (m) of identical receptor spots, of which only 2 are shown in the model, are considered to be lying side by side over a total area of 10,000 μ^2 of ending. Since r and r_1 have been neglected, the actual value of m is inconsequential, provided the ratio $n/m = x$ is constant.

If n receptor sites become active, the response (ΔV), as measured across the first two Ranvier nodes, is given by the following equation:

$$\Delta V = \frac{0.225x}{1 + 9x} (1 - e^{-t/R_1C}) \qquad (1)$$

where

$$x = \frac{n}{m} = \text{fractions of active spots}$$

and

$$R_1 = \frac{0.05}{1 + 9x}$$

(R_1 corresponds to the parallel combination of all resistances r_u, in parallel with resistances r_a of all active spots.)

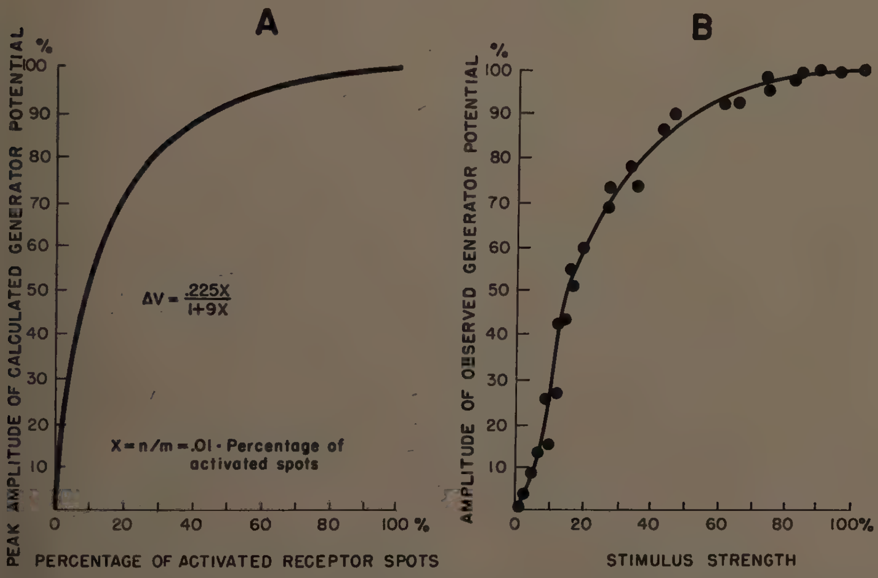

FIGURE 14. (*A*) The calculated generator potential as a function of the number of active spots. Peak response (ΔV), as calculated from EQUATION 1, is plotted against the percentage ($= 100x$) of activated receptor spots. When all available receptor spots are activated (abscissa = 100 per cent), the peak voltage is considered 100 per cent. (*B*) The observed generator potential as a function of stimulus strength. The amplitude of the generator potential from a typical experiment is plotted against the percentage of stimulus strength. The minimal stimulus strength that produces the maximal generator potential (ordinates = 100 per cent) is considered 100 per cent.

It is evident from EQUATION 1 that the response reaches its peak value as $t \to \infty$. However, since, in the slowest case, the maximal value of R_1 is 0.05 MΩ and C is 10^3 $\mu\mu$F, the response approaches to within 10^{-6} per cent of its peak value in 1 msec. In order to simplify the computation of EQUATION 1, the nodal capacities were not considered; the true response therefore will be somewhat slower. By approximating the total impedance to that of a single resistor and capacitor in parallel, the result obtained is that in the slowest case, the true response is slowed down by a factor of about 2 with respect to the response of EQUATION 1. The true response then becomes:

$$\Delta V = \frac{23}{43}\left(\frac{0.9x}{1+9x}\right)\left(\frac{1}{p-1}\right)\left\{\frac{20}{43}(p-1) - p\,\frac{e^{-t/\tau_2}}{43}\left[20 + \frac{23}{p-1}\right] + \frac{23}{43}\,p\,\frac{t}{\tau_2}\,e^{-t/\tau_2} + e^{-t/\tau_1}\left[1 + \frac{23}{43}\,\frac{1}{p-1}\right]\right\} \tag{2}$$

where $\tau_1 = R_1C$ (R_1 having the same value as in EQUATION 1); and τ_2 is the approximated time constant (12 μsec.) of one of the repeated sections terminated in the parallel resistance and capacitance that approximate the impedance of the repeated nodal sections; and $p = \frac{\tau_2}{\tau_1}$.

The main point at issue, namely, the question whether spatial summation of electric activity of all-or-nothing sites can account for the observed behavior of generator potential, is clarified if EQUATION 1 is solved for various values of x. It is then found that ΔV, that is, the amplitude of generator potential integrated at the node, as well as its rate of rise, increase as a function of the number of active receptor spots. In FIGURE 14*a*, the peak response ($t \longrightarrow \infty$) is plotted as function of the fraction (x) of active receptor sites. The percentage values of the abscissa are multiples of x. There is good resemblance between this theoretical curve and that describing a typical stimulus strength generator potential relation obtained experimentally (FIGURE 14*b*). This is true not only for the peak voltages; there would be also a range of approximate linearity in case the duration of active state was too short to allow ΔV to reach its peak value. Moreover, both curves show a plateau range as the fraction of active receptor sites or the stimulus strength approach maximal values.

CONCLUSIONS

It now seems possible to give the following picture of the generation of electric activity in the receptor. Functionally independent transducer (receptor) sites are scattered all over the nonmyelinated ending. A weak mechanical stimulus activates (depolarizes) a fraction of the total number of receptor sites. The activation is restricted to those sites that have been mechanically activated, but does not spread to adjacent sites of the ending by local-circuit excitation. As the strength of the stimulus is increased, a larger area of ending is deformed, and thereby a progressively increasing fraction of receptor sites is activated. The generator potential is built up by spatial summation of the electric activity of the receptor sites. Stepless gradation of generator potentials with increasing stimulus strength is, at least in part, a consequence of the spatial summation of the sites. Current flows between the active receptor sites of the ending and the Ranvier node adjacent to the ending. When the total current flowing between node and active receptor sites reaches a certain critical magnitude, an all-or-nothing potential is discharged at the node that may propagate along the afferent myelinated axon of the sense organ.

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MOLECULAR ASPECTS OF VISUAL EXCITATION*

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The main purpose of this contribution is to examine at the molecular level the manner in which the absorption of light acts upon the photosensitive pigments of the eye. However, we should like to go further and begin to understand how these initial changes are translated into a nervous excitation. The present status of this situation has recently been reviewed by Wald.[1]

Unfortunately, nothing is as yet known about the molecular mechanisms involved in the excitation of any end organ. We know, however, that all such excitations result eventually in a nervous impulse that involves electric fluctuations brought about by movements of ions. A number of contributions to this monograph deal with these phenomena. We shall therefore direct our attention to the manner in which the molecular changes induced by light might produce such ion currents.

In order to approach these problems, it helps to be acquainted with the structure, as close to the molecular level as possible, of the apparatus within which the process of excitation takes place. In the case of visual excitation these are the rods and cones of the retina. Each of these consists of an inner and outer segment. The inner segment contains the nucleus and carries on the vegetative functions of the cell; the outer segment contains the visual pigments and is specialized for photoreception. The outer segment of a vertebrate rod or cone consists of a stack of one to several thousand disks, regularly spaced, and each made up of 1 or 2 layers or membranes. Each membrane is about 40 to 160 Å thick, depending upon the species of animal.[2] On the basis of their properties in polarized light, W. J. Schmidt showed, twenty years ago, that vertebrate rods and cones contain alternate layers of protein and lipid,[3] and it is likely that the membranes made visible in the electron microscope[2] represent one or the other of these components.

Within this highly organized, regular structure, the molecules of visual pigment are oriented in a definite geometry. Their chromophores lie in planes perpendicular to the long axis of the rod,[3, 4] that is, within or parallel to the membranes. A rhodopsin molecule of molecular weight about 40,000, if spherical, has a diameter of about 40 Å. Its dimensions are therefore of the same order of magnitude as the thickness of the rod membranes. It is important to realize, further, that rhodopsin is a major structural component of the rod outer limb, constituting about 14 per cent of its dry weight in cattle and about

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35 per cent in frogs. This represents about 22 and 60 per cent of the respective nonlipid dry weights.[5]

The changes that light induces in the visual pigment molecules, therefore, occur within the framework of a highly organized structure that has many of the properties of the solid state, and hence in definite relationships of configuration and charge with their molecular environment.

Let us now briefly summarize the chemistry of the visual pigments. TABLE 1 shows the precursors of four visual pigments and the products of their bleaching by light. Of these 4 pigments, 2 are found in rods and 2 in cones. All 4 are synthesized by the combination of colorless proteins, members of the family of opsins, with the 11-*cis* isomer of retinene$_1$ or retinene$_2$, called neo-*b*, and all 4 bleach in the light to opsin and the all-*trans* isomer of the appropriate retinene. The configurations of the various stereoisomers of retinene are summarized in FIGURE 1.

TABLE 1

SUMMARY OF THE SYNTHESIS AND PHOTOCHEMICAL BLEACHING OF VISUAL PIGMENTS*

Visual pigment	Precursors		Products of bleaching	
	Retinene	Opsin	Retinene	Opsin
Rhodopsin	11-*cis*-retinene$_1$	Rod	All-*trans*-retinene$_1$	Rod
Porphyropsin	11-*cis*-retinene$_2$	Rod	All-*trans*-retinene$_2$	Rod
Iodopsin	11-*cis*-retinene$_1$	Cone	All-*trans*-retinene$_1$	Cone
Cyanopsin	11-*cis* retinene$_2$	Cone	All-*trans* retinene$_2$	Cone

* The 4 visual pigments are synthesized by the combination of 2 types of opsin, rod or cone, with the 11-*cis* (neo-*b*) isomer of 2 types of retinene: retinene$_1$ or retinene$_2$. In the light, they bleach to opsin and the all-*trans* isomer of the retinene. The reactions have recently been reviewed in greater detail elsewhere.[1, 6]

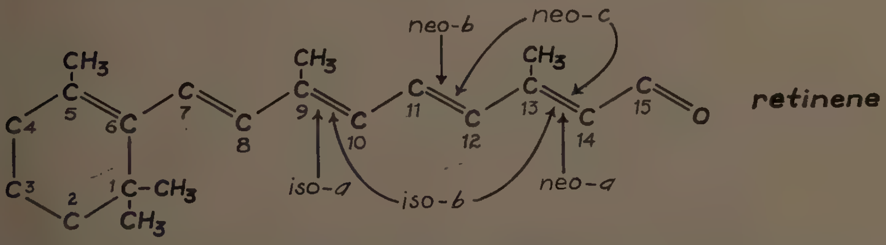


FIGURE 1. Chemical formula of retinene$_1$, showing the system of numbering carbon atoms. The structure as shown has the all-*trans* configuration. Arrows indicate the double bonds that are in *cis* configuration in the various *cis* isomers. The structures and numbering system are the same for retinene$_2$, which differs from retinene$_1$ only in possessing an additional double bond, joining carbon atoms 3 and 4 in the β-ionone ring.

The opsins are stereospecific in their requirement for the 11-*cis* isomer of retinene. This is a peculiar isomer, being not only bent, as is usual with *cis* isomers (FIGURE 2), but also twisted, since the 11-*cis* linkage is sterically hindered. The opsins fail to react with any other isomer of retinene, except one, 9-*cis* (iso-*a*), with which they form the so-called isopigments. The isopigments differ from the visual pigments in their absorption spectra, and have not been found in organisms. Bleaching by light is also stereospecific, in that it always yields primarily the all-*trans* isomer; and this is true of the isopigments as well as the visual pigments. FIGURE 2 shows the shapes of the three isomers

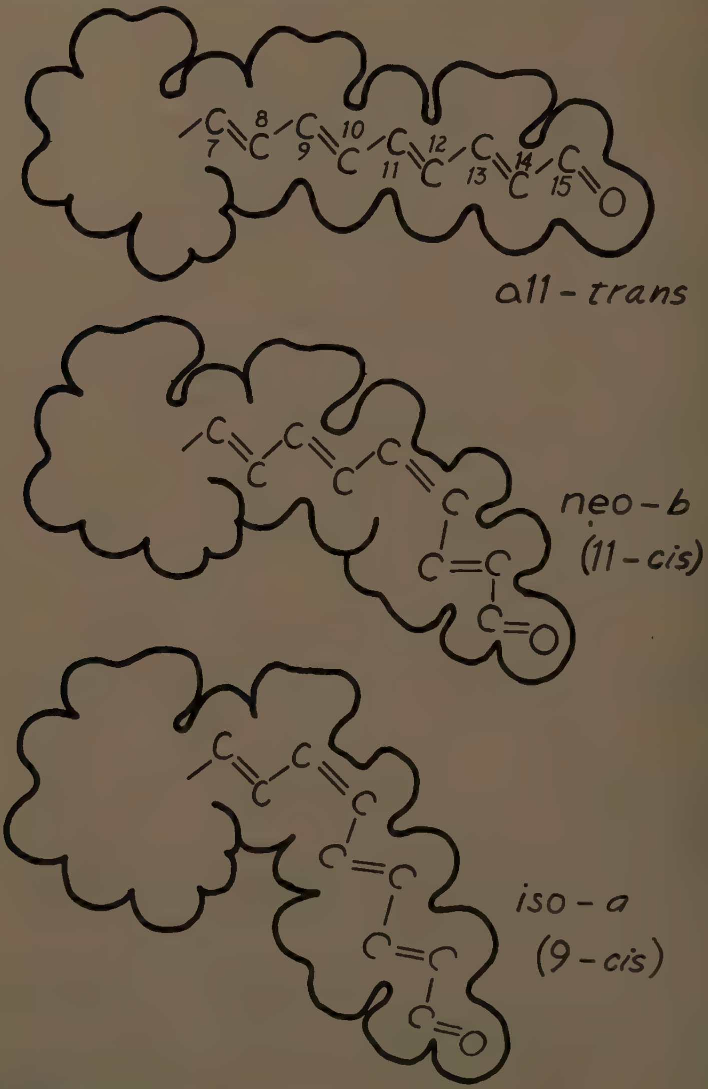
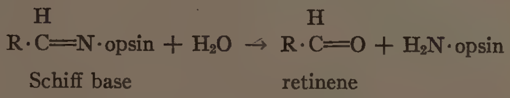


FIGURE 2. Shapes of all-*trans*, 11-*cis* (neo-*b*), and 9-*cis* (iso-*a*) retinene. The all-*trans* isomer is straight. A *cis* linkage introduces a bend into the molecule. In addition, the 11-*cis* linkage encounters steric hindrance and therefore twists the molecule out of the plane in a manner not shown in the diagram.

of retinene with which we are chiefly concerned: all-*trans*, 11-*cis* (neo-*b*), and 9-*cis* (iso-*a*).

We see, therefore, that the chromophores of all the known visual pigments have the same steric configuration, based on the bent and twisted shape of

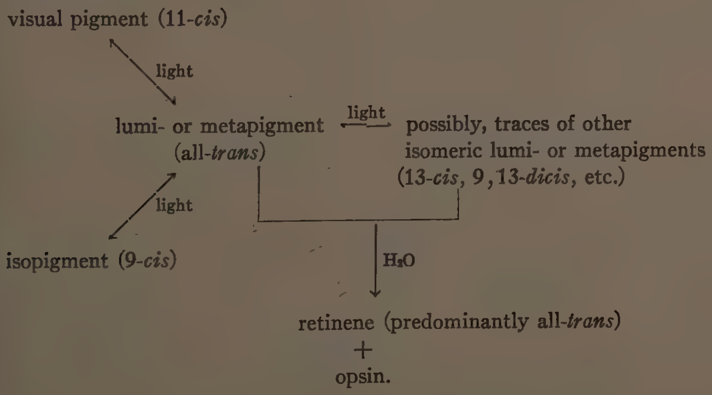
11-*cis* retinene. They fit onto opsin in a definite geometric relationship, which stabilizes the linkage between them.[6, 7] *The only direct effect of light on the visual pigments is the stereoisomerization of their chromophores to the all-trans configuration*, thus forming all-*trans* chromoproteins: the *lumi-* and *metapigments*.[7, 8] This change in the molecular geometry of the chromophore destroys its steric fit to opsin and labilizes the linkage between the two, resulting ultimately in the hydrolysis of the Schiff-base linkage that joins the aldehyde group of retinene with an amino group of opsin:[9]



$$\underset{\text{Schiff base}}{R\cdot\overset{H}{C}{=}N\cdot\text{opsin}} + H_2O \rightarrow \underset{\text{retinene}}{R\cdot\overset{H}{C}{=}O} + H_2N\cdot\text{opsin}$$

The conversion of metapigments to retinene and opsin involves the large loss of color that is bleaching. This is a characteristic of all known vertebrate metapigments, but is not part of the visual cycle of cephalopods—the squid,[10] octopus, and cuttlefish[11]—or lobsters.[12] Visual excitation in these invertebrates therefore *must* be triggered by one of the earlier reactions, and even in vertebrates it has such a short time lag that it must precede bleaching.[13] These relationships are summarized in FIGURE 3.

As may be seen from this figure, light can enter the reaction sequence a second time by isomerizing the all-*trans* chromophore of the lumi- or metapigment to *cis* configurations, including 11-*cis* and 9-*cis*, and thus regenerate visual pigment and produce some isopigment. Prolonged irradiation under conditions in which the lumi- or metapigment absorbs light therefore always yields not a single product but stereoisomeric mixtures of chromoproteins, including the all-*trans* lumi- or metapigment, the 11-*cis* visual pigment, and the 9-*cis* isopigment.[7, 8] These reactions may be summarized[8, 14] as follows:


visual pigment (11-*cis*)
light
lumi- or metapigment (all-*trans*)
light
possibly, traces of other isomeric lumi- or metapigments (13-*cis*, 9,13-*dicis*, etc.)
light
isopigment (9-*cis*)
H_2O
retinene (predominantly all-*trans*) + opsin.

We have recently examined lumi- and metarhodopsins from a number of vertebrates and invertebrates, and also lumi- and metaiodopsin, derived from the cones of the chicken retina.[14-16] Our results may be summarized as follows.

Light isomerizes the chromophores of all these pigments, yielding lumipigments, which are stable only below about −45° C. Above this temperature, in light or darkness, the lumipigments go over spontaneously to metapigments.

The latter vary somewhat in their thermal stabilities. Thus, vertebrate metarhodopsins and chicken metaiodopsin are relatively unstable and hydrolyze at temperatures above about −20° C. to all-*trans* retinene and opsin. On the other hand, the invertebrate metarhodopsins that have been examined are stable at least up to about +20° C., and probably do not hydrolyze under physiological conditions.[10-12, 15]

The absorption properties of the lumi- and metapigments that we have examined are summarized and compared with those of the corresponding visual pigments in FIGURES 4, 5, and 6 and TABLE 2. Some time ago Dartnall[17] pointed out that the absorption spectra of the visual pigments are very similar in shape when plotted on a frequency basis. We can now say the same for the lumi- and metapigments, for the half-widths of the absorption bands, particularly on a frequency basis, do not vary greatly from one pigment to another or from species to species. The λ_{max}, on the other hand, varies with species in all three classes of pigment (visual, lumi- , and meta-), presumably owing to species differences in the opsins.[6, 7, 14]

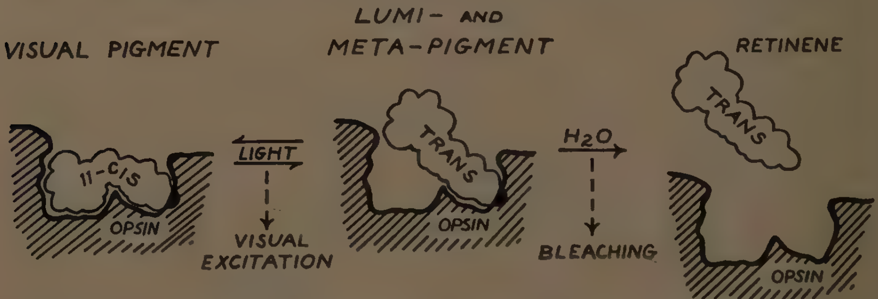


FIGURE 3. Hypothetical scheme showing the effect of light on visual pigments. The 11-*cis* (neo-*b*) chromophore fits the chromophoric site on opsin. Light isomerizes the chromophore to the all-*trans* configuration, thereby disrupting this fit and yielding the all-*trans* lumi- and metapigments. Visual excitation is probably triggered in the course of these transformations. The vertebrate metapigments (metarhodopsin and metaiodopsin) are unstable, and hydrolyze above about −20° C. to all-*trans* retinene and opsin, the reaction responsible for bleaching. The invertebrate metarhodopsins that have been examined are more stable, and probably never hydrolyze under physiological conditions.

The sets of chromoproteins from any one species exhibit certain spectroscopic regularities. Thus, λ_{max} of the lumipigment is always at longer wave length than that of the corresponding metapigment. Furthermore, with the rod pigments, λ_{max} of lumirhodopsin in general lies at longer wave length than that of the corresponding rhodopsin. However, this is not the case with the chicken cone pigment. The absorption bands of lumi- and metaiodopsin resemble in λ_{max} (and shape) those of the lumi- and metarhodopsins, and lie at considerably *shorter* wave lengths than that of iodopsin.

We have seen that the transformation of visual pigments to lumipigments involves the stereoisomerization of the *cis* chromophore by light. We know also that the conversion of the metapigments to retinene and opsin involves the hydrolysis of the Schiff base linkage that joins the chromophore to opsin in all three classes of chromoproteins: visual pigments, lumipigments, and metapigments.[6, 7] What chemical reaction underlies the conversion of lumi- to metapigments?

To answer this question we must resort to less direct evidence than is available for the other 2 reactions. At the temperatures at which the lumipigments are stable, the solvent system (a 2:1 mixture of glycerol with 2 per cent aqueous

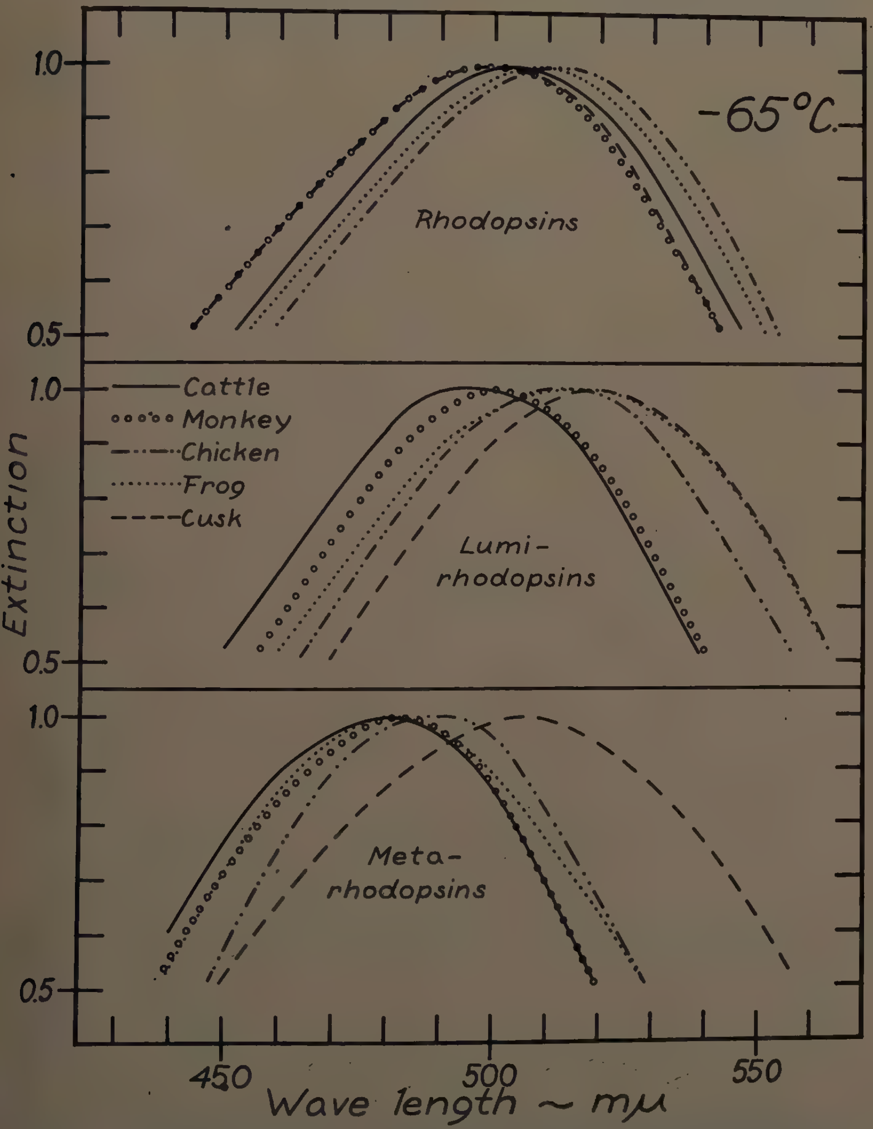


FIGURE 4. Absorption spectra of rhodopsins, lumirhodopsins, and metarhodopsins from 5 species of vertebrates, measured in 2:1 glycerol-2 per cent digitonin at −65° C. These spectra are obtained as previously described.[14] For comparison, all are brought to an extinction of 1.0 at λ_{max}.

digitonin) forms a rigid liquid, or glass, that "melts" over the temperature range in which the lumipigments change to metapigments. In this glass at low temperatures (below about −45° C.) opsin appears to have little freedom to change its configuration. At such temperatures one can therefore isolate the primary photochemical event: the isomerization of the chromophore. However, as the temperature is raised these restrictions are removed and opsin can rearrange to a new configuration. We believe this to be the transformation involved in the conversion of lumipigments to metapigments.

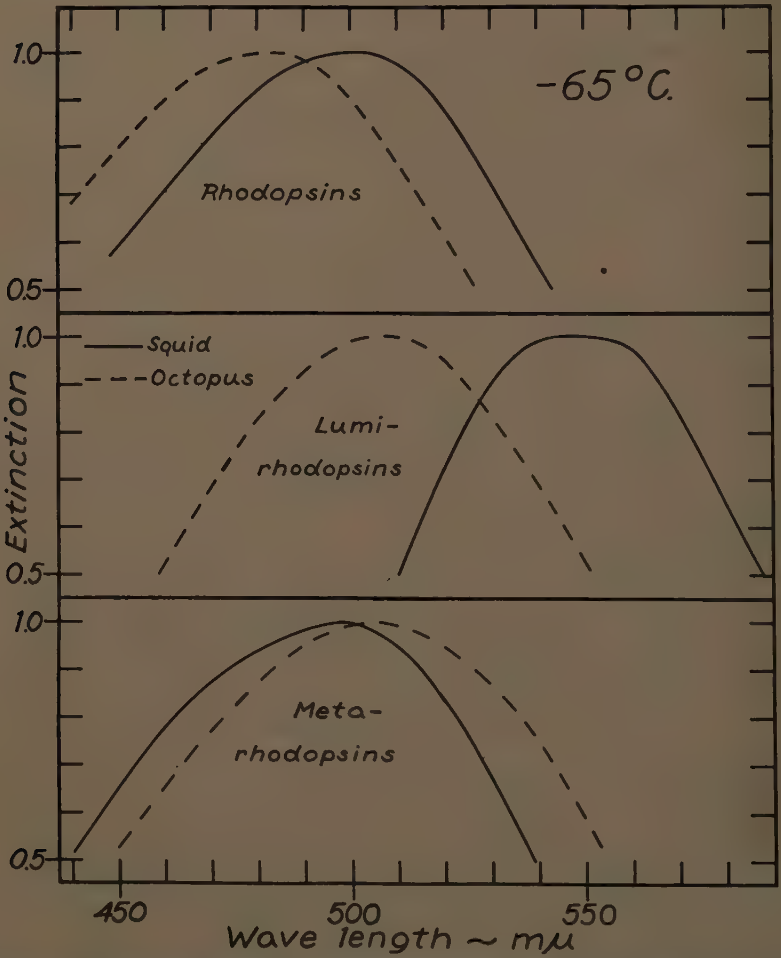


FIGURE 5. Absorption spectra of rhodopsins, lumirhodopsins, and metarhodopsins from 2 species of cephalopod (squid and octopus) measured in 2:1 glycerol-2 per cent digitonin at −65° C., as previously described.[15] For comparison, all are brought to an extinction of 1.0 at λ_{max}.

This assumption is based on the following considerations. Numerous lines of evidence suggest that the configuration of the protein is different in rhodopsin and opsin. Thus, the conversion of rhodopsin to opsin (plus retinene) is accompanied by a change in isoelectric point[18] and by the exposure of a number of reactive groups that are masked in rhodopsin: 2 to 3 sulfhydryl groups,[19] an acid-binding group of about pK 6.6,[18] and, more slowly, an H^+-liberating

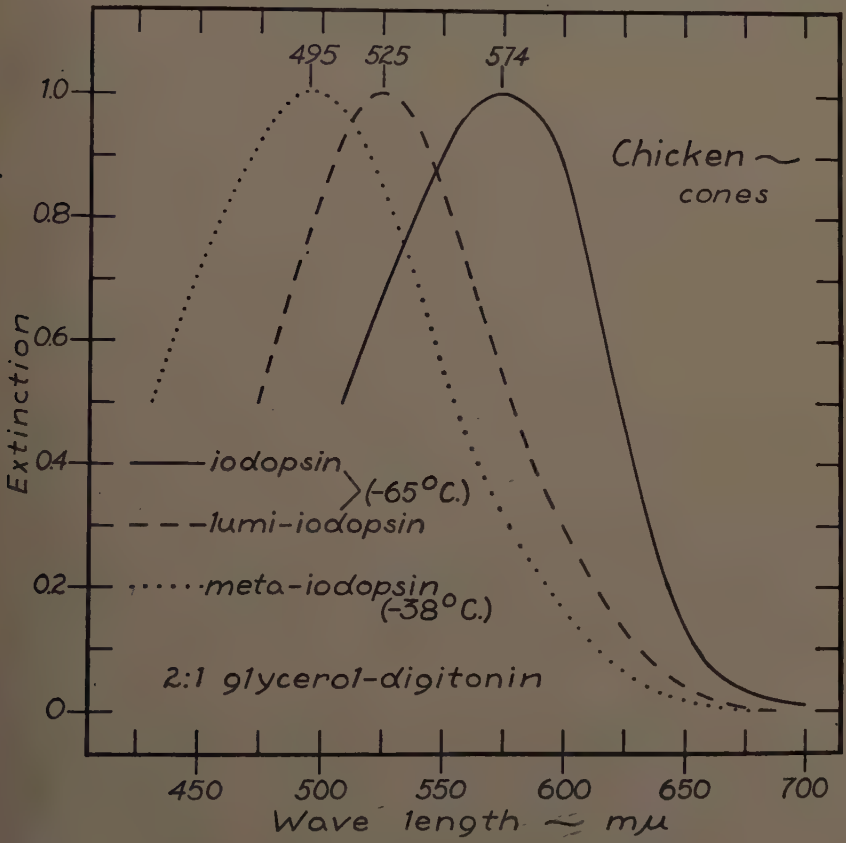


FIGURE 6. Absorption spectra of chicken cone chromoproteins (iodopsin, lumi-iodopsin and metaiodopsin) in 2:1 glycerol-2 per cent digitonin. The absorption spectra of iodopsin and lumi-iodopsin were measured at −65° C., those of metaiodopsin at −38° C. Each curve is an average from 2 sets of data, obtained as previously described.[16] The molar extinction coefficients of the chicken cone chromoproteins have not been determined, and the spectra are brought arbitrarily to an extinction of 1.0 at λ_{max}.

group.[18] Furthermore, opsin is denatured much more readily than rhodopsin, both by acids and bases,[20] and by heat.[21]

Opsin is about 100 times larger than retinene, on a weight basis. The actual binding site for retinene must therefore occupy a quite restricted region on opsin, and the exposure of this region in itself is unlikely to have such broad consequences. It seems probable that the conversion of rhodopsin to opsin carries with it a more general change in the structure of the protein, a loosening of its fabric, which exposes reactive groups and increases the ease of denaturation.

Let us try to pinpoint the change in configuration of opsin more precisely in the sequence of reactions that occur during the conversion of rhodopsin to retinene and opsin. Observations with the squid chromoproteins have revealed differences in the stabilities of rhodopsin and metarhodopsin, which suggest a greater availability of reactive groups and a loosening of the protein structure in metarhodopsin as compared with rhodopsin.[10] Furthermore, in octopus and squid, the transformation of lumirhodopsin to metarhodopsin is accompanied by the exposure of an H^+-liberating group, presumably the Schiff-base linkage between the chromophore and opsin.[15] Its exposure is responsible for the ap-

TABLE 2

ABSORPTION PROPERTIES OF VISUAL CHROMOPROTEINS FROM SEVERAL VERTEBRATES AND INVERTEBRATES*

Visual cells	Species	Pigments	λ_{max} (mμ)	ϵ_{max} (liter/mole-cm.)	$\lambda_{1/2}$ (mμ)	$\nu_{1/2}$ (10^{14} sec.$^{-1}$)
Rods	Monkey	Rhodopsin	500	49,000	100	1.26
		Lumi	500	56,000	87	1.05
		Meta	482	48,000	83	1.09
	Cattle	Rhodopsin	504	47,000	96	1.17
		Lumi	497	54,000	92	1.13
		Meta	481	50,000	86	1.14
	Chicken	Rhodopsin	512	47,000	96	1.13
		Lumi	513	49,000	94	1.09
		Meta	489	38,000	83	1.05
	Bullfrog	Rhodopsin	508	46,000	98	1.17
		Lumi	515	43,500	106	1.23
		Meta	482	49,000	91	1.18
	Cusk	Rhodopsin	500	52,000	100	1.26
		Lumi	519	54,000	96	1.09
		Meta	507	39,000	108	1.30
Cones	Chicken†	Iodopsin	574		115	1.09
		Lumi	525		104	1.14
		Meta‡	495		124	1.55
Cephalopod rods	Squid	Rhodopsin	500	48,000	102	1.28
		Lumi	550	64,000	83	0.84
		Meta§	500	65,500	102	1.29
	Octopus	Rhodopsin	483	43,000	98	1.31
		Lumi	508	38,000	94	1.31
		Meta§	505	63,000	105	1.29

* Measured in 2:1 glycerol-2 per cent digitonin at −65° C.; wave length of maximal absorption (λ_{max}); molar extinction coefficient at that wave length (ϵ_{max}); half width of the absorption band (that is, the width of the band when the extinctions are half maximal) measured on the basis of wave length ($\lambda_{1/2}$) and frequency ($\nu_{1/2}$). The manner in which these data are obtained has been described elsewhere.[14-16]

† The molar extinction coefficients of the chicken cone chromoproteins have not been determined.

‡ The absorption properties of chicken metaiodopsin have been measured at −38° C., which may account for the width of the absorption band.

§ Only the properties of the acid metarhodopsins[10, 11] are given here, since it is this form of the cephalopod metarhodopsins that is homologous with the metapigments of vertebrates.[6, 7, 11, 15] pearance of *p*H indicator properties in the cephalopod metarhodopsins,[10, 11] which are masked in the lumirhodopsins.[15]

We have shown above that the transformation of visual pigment to lumipigment involves probably nothing more than the photoisomerization of its chromophore. The thermal rearrangement of opsin must therefore occur subsequently, either during the transformation of lumi- to metapigment, the hydrolysis of metapigment, or at both stages. Clearly, some of the changes in protein configuration take place during the conversion of lumi- to metarhodopsin, whatever further changes may occur subsequently during the hydrolysis of metarhodopsin to retinene and opsin.

In summary, therefore, we are suggesting that the polypeptide chain of opsin can fluctuate between different configurations, one of which prevails in the presence of the 11-*cis* chromophore (visual pigment), another when combined with the all-*trans* chromophore (metapigment), and perhaps a third one in the absence of a chromophore (opsin). However, when the *cis* chromophore of the visual pigment is isomerized to all-*trans* (lumipigment) in a rigid solvent at low temperatures, the configuration of the protein, although unstable, is unable to rearrange until the temperature is raised sufficiently to render the system more fluid. Similar configurational changes have recently been invoked with reference to a number of other proteins and enzymes by Linderstrøm-Lang and Schellman,[22] and by Lumry.[23]

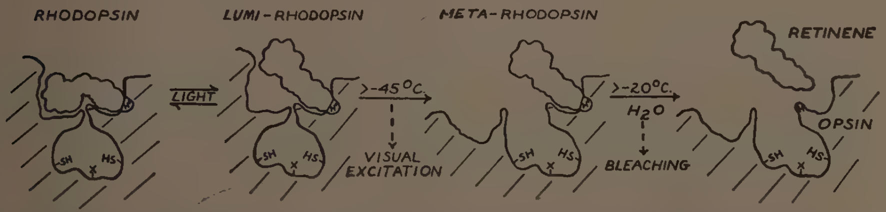


FIGURE 7. Summary diagram of the action of light on rhodopsin. When the 11-*cis* chromophore of rhodopsin is isomerized by light, forming all-*trans* lumirhodopsin, its steric relationship to opsin is changed. This causes the opsin to change its configuration and form metarhodopsin as soon as the temperature is raised sufficiently to permit such structural rearrangements. The rearrangement of opsin from lumi- to metarhodopsin exposes reactive groups, which may cause an electric disturbance in the rod outer limb and thus trigger an excitation. Furthermore, vertebrate metarhodopsins are unstable and hydrolyze to all-*trans* retinene and opsin, the reaction responsible for bleaching (compare FIGURE 3).

In closing, let us return to our initial question of how the molecular changes initiated in the visual pigments by light can trigger a nervous excitation. It should be clear from what has been said that little is to be expected in this connection from the transformation of visual pigment to lumipigment. Likewise, the hydrolysis of metapigment can be ruled out, since it is too slow to trigger an excitation in vertebrates and probably does not occur in cephalopods and lobsters. This leaves the conversion of lumi- to metapigment as the most likely trigger reaction. These considerations are summarized diagrammatically in FIGURE 7.

The unmasking of specific reactive sites or the change in general charge distribution associated with the rearrangement of the protein must produce transient localized disturbances in the organized pattern of charges in the rod and cone outer limb. If these are of sufficient magnitude—a large "if"—they may initiate a flow of ions adequate to trigger the discharge of the visual cell.

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MUSCULAR CONTRACTION

INTRODUCTORY REMARKS

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During the thirteen years that have passed since the first publication on muscular contraction[1] of The New York Academy of Sciences, great advances have been made in the many facets of our knowledge of muscle fiber.

This monograph deals with several of the currently most critical problems of fundamental muscle research and these, furthermore, by deliberate intention are discussed in great part in respect to results obtained on the intact muscle or muscle fiber. Only secondarily is evidence included from work on substances extracted from muscle and the "models" that can be reconstructed from them. There is no intention in this relative exclusion of investigations on extractives from muscle to deprecate the great intrinsic interest and importance of such studies. However, such studies at best can suggest only what is true for the actual muscle fiber; in the last analysis, the nature of the mechanisms of this protoplasmic system must be determined from results of studies made as directly as possible on the actual physiology of that system.

Thus, the first part of this publication deals with what seems, as revealed by electron microscopy and related techniques, to be the basic organization of the actin and myosin fibrils into a contractile system and a theory of the mechanochemical behavior of this structure. True, the primary configurational observations of this work have not been made on the living but on the dead fiber. Nevertheless, muscle fibers are involved, and it is difficult to see how the observed interdigitating organization of the contractile fibrils could have been conceived from investigations of only extractives obtained from wholly destroyed muscle cells. The second part is concerned with the coupling of the electric events of the membrane to activation of the mechanical response of the internally located contractile system, and here the work is physiological throughout. In any case, research on electromechanical coupling must be done on the living fiber, since no model of it has been developed thus far. As for the third part, although it includes research on relaxation systems as studied in test-tube mixtures containing extractives, the main emphasis is on the problem of mechanochemistry of the intact, living muscle. Here especially there is very great need for studies of the chemical changes of physiologically functioning muscle. It is well known that the conclusion derived from research on extracted systems—that the energy for contraction is obtained from the breakdown of adenosinetriphosphate—is questionable for physiological contraction, since the evidence obtained to-date on the living muscle indicates, although not with absolute certainty, that this nucleotide does not break down even during a contraction of a fresh muscle or, if it does, that it does so too late to supply the energy for work done during the contraction period. It is clear that this contradiction and, in general, the question of the source of energy for contraction, can be resolved only by more work, such as that reported in this monograph, on the mechanochemical relations of the intact muscle.

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In view of the predominantly physiological orientation of this monograph, it was very appropriate that Wallace O. Fenn (University of Rochester School of Medicine and Dentistry, Rochester, N. Y.), a physiologist, acted as honorary chairman of the conference on which this monograph is based. Fenn obtained his doctoral degree in 1919 in plant physiology, but it was not long before he was attracted to the study of the much more overtly moving animal. As early as 1923[2] and 1924,[3] he was already in print with his work done in the laboratory of A. V. Hill on the shortening heat of contraction: now classically called the Fenn effect. I quote from his article "Introduction to Muscle Physiology"[4]: "The muscle is a machine which goes. It can be made to 'run' at will. As a gadget which works it has an obvious fascination for any boy or girl. It has a similar fascination for physiologists, the what-makes-it-go boys of biology. It is a lasting fascination and formidable challenge, for as yet we have no adequate comprehension of its machinery." With little change, this still aptly expresses his interest, and ours, in muscle today. May it also play a role in activating him and all students of muscle to new efforts in the attempt to comprehend the mechanisms of muscular contraction.

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Part I. Muscle Fibers and Fibrils

THE STRUCTURAL BASIS OF THE CONTRACTION MECHANISM IN STRIATED MUSCLE

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Knowledge of the detailed way in which the molecules of the structural proteins in muscle are assembled into a system that will exhibit contractility on the macroscopic level should enable us to do two things. First, it should help us to see how the elementary contractile events occurring at the molecular level are integrated to produce large-scale shortening of the muscle. Apart from its intrinsic interest, this may help us to understand to what extent muscles having different mechanical characteristics may be constructed from essentially similar components and have the same basic principles of operation. Second, it should enable detailed knowledge of the macroscopic properties of muscle, such as its mechanical and thermal behavior, to be used to define more closely the characteristics that the elementary processes must possess. We can then attempt to correlate those characteristics with the chemical and physical properties of the muscle proteins.

During the last few years a considerable body of evidence has been accumulated to support the idea that the contractile structure of striated muscle is built up of a succession of overlapping arrays of submicroscopic filaments containing the two principal structural proteins, myosin and actin. These two proteins seem to be located in separate arrays, alternating along the length of the fibrils, thus giving rise to the well-known band pattern. Changes in the length of the muscle, whether active or passive, take place when actin and myosin filaments slide past each other, so that the extent of overlap of the arrays is altered. Cross-bridges project out sideways at short intervals along the myosin filaments (6 bridges every 400 Å), and it appears likely that their interaction with the neighboring actin filaments controls the mechanical state of the muscle in the following way. When they are not attached to the actin, the filaments are free to slide past each other, and the muscle can be stretched easily; that is, it is "relaxed." If the bridges become permanently attached to the actin filaments, no sliding can occur, the muscle is rigid and inextensible, and it is said to be in rigor. If they are intermittently attached to the actin filaments, then some unknown process causes a relative sliding movement to take place between the two sets of filaments, and contraction occurs.

The evidence for these views has been described in a number of papers recently and has been dealt with at length, together with its implications, in several review articles (Hanson and H. E. Huxley, 1955; A. F. Huxley, 1957; H. E. Huxley and Hanson, 1959; H. E. Huxley, 1959). For convenience TABLE 1 lists the principal sources for the various pieces of experimental evidence.

The appearance of the structure in electron micrographs is shown in FIGURES 1, 2, and 3, and the changes associated with shortening are shown diagrammatically in FIGURE 4.

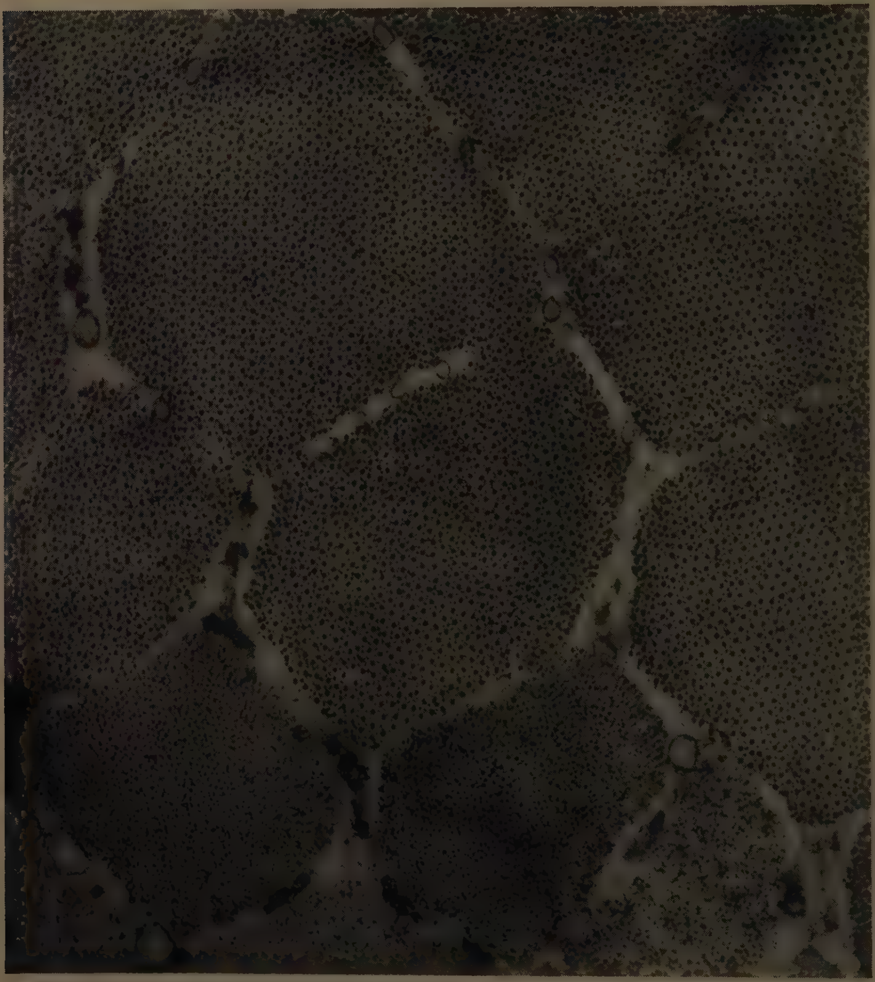

FIGURE 1. Cross-section through a number of myofibrils of rabbit psoas muscle, showing very regular double array of filaments in many areas. ×58,000.

Dissenting views have been expressed by a number of authors (Hodge, 1955, 1956; Szent-Györgyi *et al.*, 1955; Spiro, 1956*a*, 1956*b*; Sjöstrand & Andersson-Cedergren, 1956, 1957). In the main, the various objections raised have now been answered in the papers listed in TABLE 1. Some points of difference still exist, but we regard the principal features of the scheme as being sufficiently well established for it to serve as a basis for discussion of detailed theories of contraction.

Perhaps the most important feature of the scheme, certainly one that distinguishes it sharply from most other models, is the repetitive, cyclic operation of the active sites throughout the time that the muscle is contracting. Even during a single twitch of a frog sartorius muscle at 0° C., each site will operate up to approximately five times; on each occasion, a molecule of energy-rich phosphate will be broken down at some unknown point in the cycle of opera-

TABLE 1
PRINCIPAL SOURCES OF EXPERIMENTAL EVIDENCE

(1) Construction of muscle fibrils from overlapping arrays of filaments.		
	Hanson and H. E. Huxley	1953
	H. E. Huxley	1953*b*
	H. E. Huxley	1957
(2) Localization of actin and myosin in separate arrays of filaments.		
	Hasselbach	1953
	Hanson and H. E. Huxley	1953
	Hanson and H. E. Huxley	1955
	H. E. Huxley and Hanson	1957*a*
	Hanson and H. E. Huxley	1957
	Corsi and Perry	1958
(3) Sliding of filaments during contraction and stretch.		
	A. F. Huxley and Niedergerke	1954
	H. E. Huxley and Hanson	1954
	Hanson	1956
	H. E. Huxley	1957
(4) Detailed appearance of filaments as studied in electron microscope.		
	H. E. Huxley	1953*b*
	H. E. Huxley and Hanson	1957*b*
	H. E. Huxley	1957
(5) X-ray diffraction evidence for presence of double array of filaments and for invariance of filament length.		
	H. E. Huxley	1951
	H. E. Huxley	1952
	H. E. Huxley	1953*a*

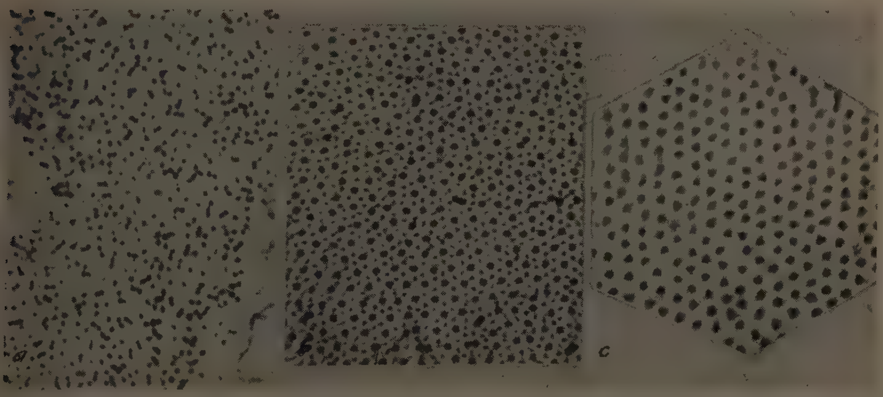

FIGURE 2. Cross-sections through striated muscle at different positions in the sarcomere: (*a*) through the I-band, showing thin filaments only; (*b*) through the denser regions of the A-band, on either side of the H-zone, showing large and small filaments together; (*c*) through the H-zone in center of A-band, showing large filaments only. ×66,000.

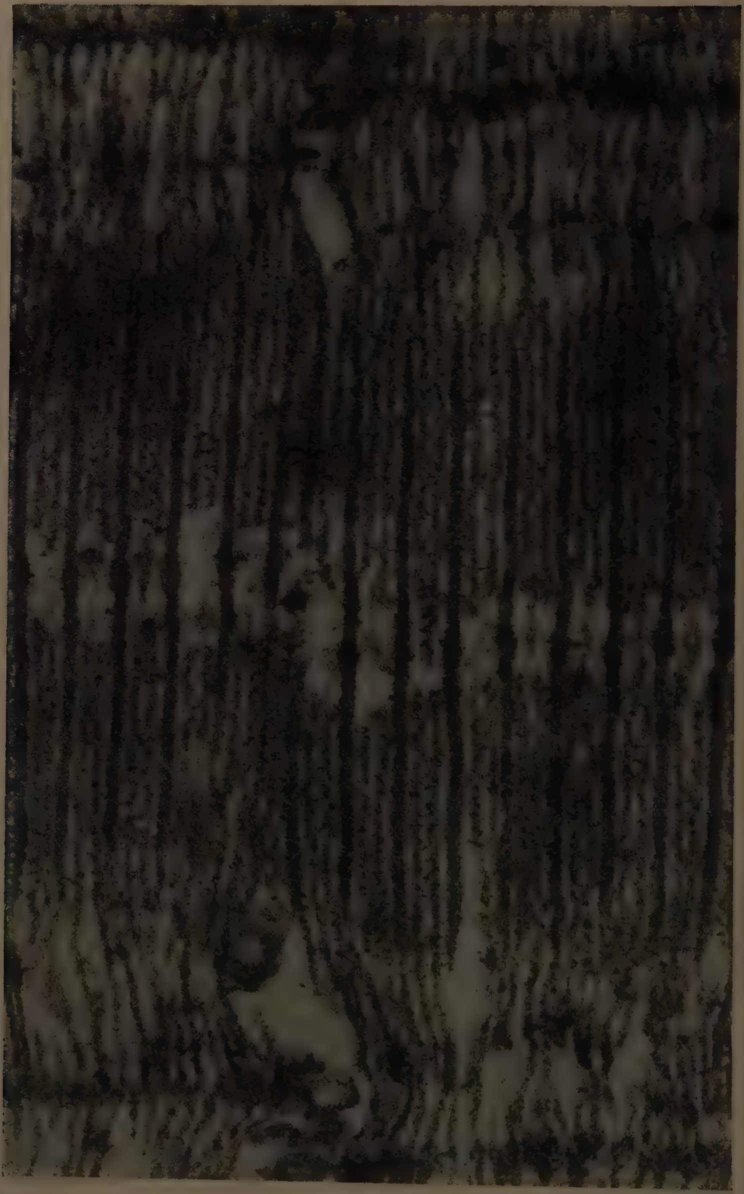

FIGURE 3. Very thin (~150 Å) longitudinal section through rabbit psoas muscle, showing the overlapping of the two types of filaments. Pairs of thin filaments are seen between the thicker ones because of the geometry of the lattice; cross-bridges extend between the two types of filament. From H. E. Huxley, 1957; reproduced with permission from the *Journal of Biophysical and Biochemical Cytology.* ×140,000.

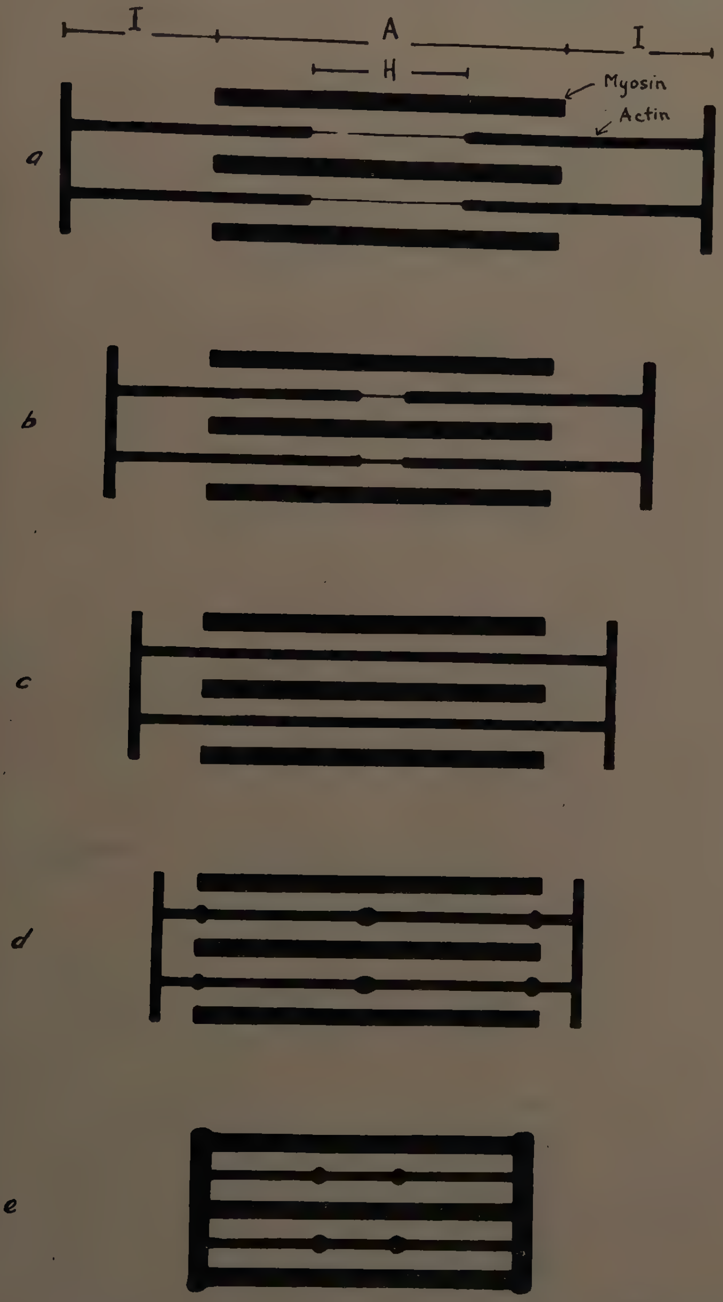

FIGURE 4. Diagram illustrating arrangement of myosin and actin filaments in striated muscle at various lengths. (*a*) Stretched; (*b*) rest-length; (*c*), (*d*), (*e*) shortened by successively greater amounts. The basic process is one of sliding, but some folding or crumpling of filaments at their ends may take place at higher degrees of shortening.

tion at the site, and the site will contribute to the shortening or tension development of the muscle.

The manner in which the active sites, presumably localized on the cross-bridges, exert a mechanical effect on the structure during contraction, remains a matter for speculation. One possibility is that the cross-bridges (or the sites of linkage between the filaments) can move to and fro over a short range in an axial direction, either as a necessary accompaniment of enzymatic activity, or simply by Brownian motion (A. F. Huxley, 1957), and, by attaching to and detaching from the actin filaments at suitable points in their cycle of oscillation, can exert an undirectional pull on those filaments. Another possibility is that the attachment of the bridges results in some internal rearrangement of the actin molecules within the filaments; this could give rise to a repetitive length change in the actin filaments, which in turn could be arranged to produce a directional movement of those filaments past the cross-bridges (Hanson and Huxley, 1955; H. E. Huxley, 1959; H. E. Huxley and Hanson, 1959). Probably the most interesting problem now is to try and think of experiments that will give us detailed and, if possible, direct information about these elementary processes, so that we can decide between the different sorts of possibilities that exist.

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THE RELAXATION OF THE CONTRACTED ACTOMYOSIN SYSTEM

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We know several mechanisms that cause fibers of protein or of synthetic high polymers to contract. These mechanisms have become known through the researches of K. H. Meyer, T. Astbury, W. Kuhn, Mark Pryor, and A. Katchalsky. All of these mechanisms have one thing in common, namely, that the contraction of the macroscopic fiber is based on a folding or coiling of thread molecules. For this reason all of these mechanisms cannot be used to explain muscle contraction, because the ultravisible filaments within the muscle do not shorten but, instead, slide unshortened alongside each other.

Therefore, I have been asked to discuss my theory about the chemical processes, first advanced in 1956,[1] that cause filaments to slide alongside each other, according to H. E. Huxley and J. Hanson,[2-4] as well as A. F. Huxley.[5-7] I developed this theory chiefly from observations that have been made on the contraction phase of the living muscle and of isolated contractile systems. The theory is acceptable only if it is also in agreement with the observations on the relaxation phase. In the United States I have already put forward my theory explaining the contraction phase, for instance, in one of my Dunham Lectures in 1957.[8]* Since these lectures have already been published, I intend to deal primarily with the characteristics of the relaxing factor and to discuss how far the properties of the relaxation phase accord with my theory. I assume that there is agreement on this, particularly since Gergely's investigations, reported elsewhere in this monograph, and our own have revealed some new facts about the relaxing factor during the past year.

I shall first briefly explain my theory of the "sliding filaments," relating it to the contraction phase, and then I shall consider the relaxation phase and the system of the relaxing factors in greater detail.

Theory of the Sliding Filaments

The theory that I now propound has been evolved from the following observations.

(1) Fibrils are incapable of contracting and relaxing and are in a state of rigor if no adenosine triphosphate (ATP) or related nucleoside triphosphate (NTP) is present. The resistance to stretch in this case is very high.[9] Moreover, any stretching that exceeds a small percentage of the initial length leads to a tearing of the fibrils.[10-15]

(2) If ATP or a related NTP, together with Mg^{++}, is present, contraction takes place as long as the fibrils split off the terminal phosphate of the NTP.[16]

(3) If the splitting is inhibited completely or almost so by physiological[17] or pharmacological[9] means, relaxation occurs. The fibrils are incapable of contracting (Hasselbach, unpublished data).

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(4) In the presence of ATP whose splitting is inhibited, the fibrils are as soft and plastic as in the living muscle during relaxation and rest. The resistance to stretching is about 100 gm./sq. cm. of the cross section. During contraction this resistance increases between tenfold and fiftyfold. It is several kg./sq. cm. of the cross section.[9]

(5) According to H. E. Huxley and J. Hanson, the great extensibility of the fibrils during relaxation and rest is produced by the slipping of the I-filaments from the A-filaments.[4, 5]

Hence, in the presence of Mg^{++} the various NTP,[16] and especially ATP, have 2 different effects. If not split, they lower the resistance to reversible stretch; that is to say, they lower the cohesive forces between the A- and I-filaments, and the higher the ATP concentration the greater the effect. On the other hand, ATP splitting produces both contraction and an increase in resistance to stretch by more than one order of magnitude.[9] These effects of ATP splitting are the same for ATP concentrations between $\sim 10^{-4}$ and $\sim 10^{-2}$ M ATP.

It follows that the A- and I-filaments are interconnected much more strongly during contraction than during rest. The sliding of the filaments, on which contraction is based, is apparently accompanied by the production of chemical bonds between the A- and I-filaments.

Any theory that attempts to explain the chemical processes underlying the sliding of the filaments must take into account two consequences of ATP splitting. On the one hand, it must point out that ATP splitting in the presence of Mg^{++} obviously supplies the energy necessary for contraction and, on the other, that by means of ATP splitting in the presence of Mg^{++}, chemical bonds between the A- and I-filaments are produced. Such a theory becomes all the more plausible the more it makes use of chemical reactions well known in the field of biochemistry. We now know from studies of the chemistry of metabolism that ATP splitting in the presence of Mg^{++} often induces chemical reactions between substances that, without ATP splitting, do not react with each other. Further, we know that in such cases the energy produced by ATP splitting is used for chemical reactions that require energy.

An especially well-known example is the synthesis of fatty acid chains from acetate by coenzyme A (CoA) in the presence of ATP and Mg^{++}.

We apply this example to our problem in the following way.[8]

(1) We assume that, in the first stage of the sequence of reactions, one of the two fragments resulting from the splitting of ATP, either ADP or inorganic phosphate, combines with a chemical group of the I-filament. This is an analogous reaction to the first step of the synthesis of fatty acids, including a transphosphorylation of AMP from ATP to acetate.

(2) We assume an exchange of this ATP fragment with a functional group of the A-filament. Thus, from the ATP fragment-I-filament complex originates an I-filament-A-filament complex. This corresponds to the conversion of acetyl-AMP into acetyl-CoA, the second step of fatty acid synthesis.

(3) We assume that in a sequence of further reactions the first functional group of the A-filament is exchanged with a second, third, and perhaps a fourth functional group of the A-filament. If we refer this further sequence of reactions to the example of CoA, it is analogous to the reactions occurring

between the molecules of acetyl-CoA leading to the formation of aceto-acetyl CoA and to further sulfhydryl esters of CoA with still longer carbon chains.

(4) We assume that the various functional groups of the A-filament reacting successively with the active group of the I-filament are arranged in a linear periodicity.

A sequence of reactions during the course of which the active group of I-filaments successively reacts with several linearly arranged groups of A-filaments has the inevitable result that the active group travels alongside the A-filament while being shifted from the first to the last of the groups involved.

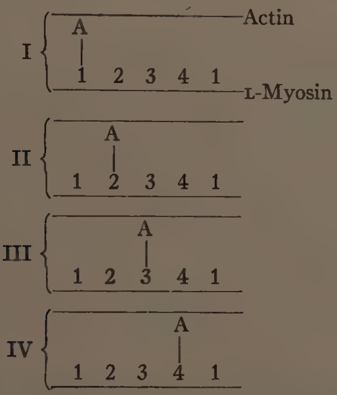

FIGURE 1. Shifting of one filament alongside another filament produced by a sequence of chemical reactions. A = active group of the actin filament; 1, 2, 3, 4 = groups of the L-myosin filament successively reacting with A.

The System of the Relaxing Factors

Now I shall adapt the proposed sequence of reactions to the particular conditions in the muscle.

H. E. Huxley has already explained that I-filaments are the same as actin filaments and that A-filaments are identical with L-myosin filaments.[18-22] We know that not only the contraction of the actomyosin systems but also the Mg-activated ATP splitting is due to an interaction between actin and L-myosin. Under physiological conditions, the rate of the Mg-activated ATP splitting by actomyosin is about 10 times as great as the splitting rate of the components actin and L-myosin when separated.[23] On the other hand, Hasselbach has shown that ATP splitting in the living muscle is activated by Mg^{++} and not by Ca^{++}. The very low content of Ca in the living muscle is completely bound to protein, whereas 90 per cent of the high Mg content is free.[24]

From investigations with labeled phosphates we have reason to assume that the ATP splitting begins with a transfer of phosphate from ATP to an energy-rich bond of the actomyosin.[25, 26]

We suppose that the phosphate residue is transferred to an acid group of the actin.

As the shift of the actin filament alongside the L-myosin filament during contraction implies performance of work, energy must be set free during this shift. This signifies that the compound formed by the reaction of actin with the first functional group of L-myosin must contain an energy-rich bond, and the compound of actin with the least group of L-myosin involved must contain a low-energy bond, so that through every step of the sequence of reactions a certain amount of energy is set free. Thus, an energy-rich bond is required between the active group of actin and the phosphate residue, and an almost equally energy-rich bond is required between the active group of actin and the first reacting group of L-myosin.

We meet this requirement by assuming, for instance, that the phosphate is bound to an acid group of the actin, and that this phosphate residue is exchanged with a sulfhydryl group of the L-myosin; for, like the bond between an acid group and phosphate, a bond between an acid group and a sulfhydryl group contains a standard energy of about 8000 cal. Furthermore, it has been well known for a long time that the SH-groups of the actomyosin complex participate in ATP splitting and in contraction. The energy contained in the first bond between actin and L-myosin is almost completely liberated if, at the conclusion of the sequence of reactions, actin and L-myosin are united by means of a normal ester bond, since such an ester bond contains only about 2000 to 3000 cal. There may be one or two bonds containing a medium amount of energy between the first and last types of bonds.

The principle of the process is represented in FIGURE 2. It is supposed that the active group of actin is passed along from a bond of the sulfhydryl group by the bond of a phenolic OH-group to a bond of an alcoholic OH-group. Subsequently, this normal ester bond is hydrolyzed, and the acid group of actin is rephosphorylated. Subsequently the sequence of reactions represented begins anew.

Since the rephosphorylated actin group is now close to the sulfhydryl group of the next period and remote from the sulfhydryl group of the first period, the active actin group is shifted alongside the next longitudinal period of the L-myosin filament if the muscle is unloaded. Consequently, as long as the muscle is unloaded, the single shifting steps without interruption amount to the visible external shortening which can be measured.

The more heavily the muscle is loaded, however, the more frequently during rephosphorylation the actin filament with its active group is withdrawn by the load into the initial position. This increases the number of shifting steps necessary for the fibril to shorten by a definite amount. Under isometric conditions a shortening that is externally visible can never be observed, because under the high isometric tension the actin filament is withdrawn into the initial position after every shifting stage.

The thermodynamic consequences arising from the theory I have propounded are discussed in my Dunham Lectures.[8] Consequently, I shall merely summarize briefly the principal result. If the filaments slide alongside each other because of the chemical principles that I have outlined, the liberation of energy per unit of time must be the greater the less the muscle is loaded. On the other hand, the liberation of energy per centimeter of shortening must be the greater the heavier the load. It is well-known that, according to A. V. Hill,[27-31] the 2 proceeding statements apply to the contraction of the living muscle.

The views I have propounded show that if some well-known reactions of

ATP utilization are appropriately arranged, it is possible to explain all the phenomena exhibited during the contraction phase by a shifting of the A- and I-filaments alongside each other. According to my theory, relaxation would depend on the cessation of ATP splitting, and hence of the interaction between actin and L-myosin filaments.

This conclusion contradicts a view that was widely accepted over a period of several years. According to this view, relaxation does not depend on the cessation of ATP splitting, but on the compensation for ATP splitting by ATP restitution.

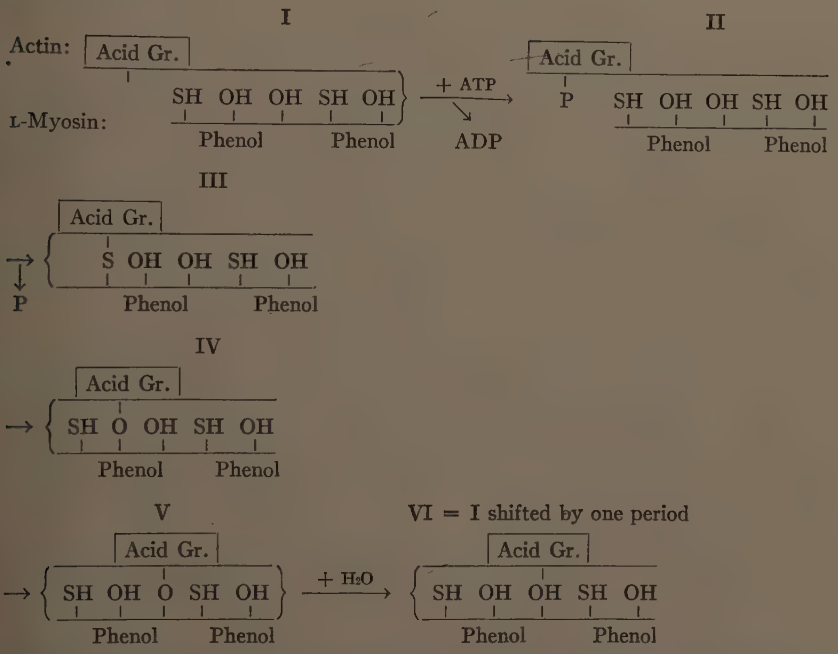

FIGURE 2. Diagram of a series of chemical reactions leading simultaneously to ATP hydrolysis and to a shifting of the molecules of the actin filaments alongside the molecules of L-myosin filaments. The Roman numerals I to VI denote successive chemical conditions and stages of shifting.

Several laboratories in England and in the United States had made the observation that the ATP-induced contraction of water-glycerin-extracted bundles of muscle fibers stops immediately when ATP-restituting systems are added to the bath. This was shown by Bendall[32] with myokinase, by Bozler,[33] and the Szent-Györgyi group[34] with phosphocreatine and phosphokinase, by Lorand[35-37] with phosphopyruvate and pyruvatekinase, and by Goodall[38] with phosphocarnosine. Therefore, for many years it was widely accepted that the relaxing factor discovered by Marsh[39] was nothing but the sum of the ATP-restituting systems contained in the sarcoplasm of the muscle. Meanwhile, this view has been disproved by Portzehl,[40] who has first shown that the relaxing factor is not localized in the sarcoplasm of the muscle, but in some granules.[40-44]

According to Hasselbach, it is a fraction of granules with a diameter of about 1000 Å.[45]

At the same time, Briggs and Portzehl showed that ATP-restituting systems cause water-glycerin-extracted muscle fibers to relax only when these fibers contained remnants of the grana factor.[46] Finally, Portzehl showed that ATP restituting systems have no relaxing effect when, instead of fibers, muscle fibrils are investigated.[47] These observations can be easily understood if one remembers certain earlier findings.

Five years ago Hasselbach showed that relaxation produced by Marsh's relaxing factor, which we shall call *granule factor*, is the more complete the higher the ATP concentration.[48] If the ATP concentration is very high, that is, two to three times as high as the physiological ATP concentration, relaxation takes place even in the absence of the factor.[49-51] We call these high ATP concentrations "supraoptimal concentrations."

From the two aforementioned findings Hasselbach and Weber[48] concluded that the effect of the relaxing factor depends solely on the fact that the range of the supraoptimal ATP concentrations is extended to physiological values by the relaxing factor. Finally, it must be taken into account that the ATP level within the fibers and the fiber bundles is much lower than outside in the bath, because the ATP diffuses into the fiber bundles only at low speed; during this diffusion ATP is split at a high speed.

The relaxing effect of ATP-restituting systems is due to the following facts.

The very low ATP concentration inside the fibers is raised to the level of the physiological ATP concentration of the bath by means of the ATP-restituting systems, because the ATP splitting is compensated by the restitution. Relaxation occurs under these conditions only when the fibers contain some granule factor able to produce relaxation in the presence of a physiological ATP concentration "inside."[42, 46] Consequently, relaxation does not occur if, due to long storage, the fibers no longer contain a relaxation factor,[42, 46] nor if fibrils are used. For the ATP concentration within the fibrils of a thickness of 1 μ is, even without an ATP-restituting system, as high as the concentration outside because of the shortness of the diffusion way.[47]

The ATP-restituting systems produce relaxation only if they raise the ATP level "inside;" the relaxing effect is not directly dependent on the restituting process. Since in the living muscle the ATP concentration inside has in any case the physiological value, the relaxing effects of the ATP-restituting systems on water-glycerin-extracted fibers and fiber bundles must be taken as artifacts.

The physiological granule factor, however, inhibits contraction and induces relaxation, not by restituting ATP, but by inhibiting ATP splitting.[17, 48] Hence, it behaves like the ATPase poisons.

However, the mechanism of the factor effect is completely different from that of the poisons, such as mersalyl. It involves an extremely complicated interplay between granules, ATP, fibrils, and a dialyzable cofactor discovered by Gergely, who reports on the discovery of this cofactor elsewhere in this monograph.

We can understand the degree to which this interplay is complicated from the following considerations and observations.

(1) It is plausible that the granules are not identical with the relaxing factor,

but that they must exchange a factor with the fibrils, for the granule with its diameter of 1000 Å cannot penetrate the fibrils. The relaxing factor, however, must reach even the innermost filaments of the fibrils.

(2) Consequently, it is not surprising that the effect of the granule suspension does not depend to any great degree on the absolute concentration of the granules, but does depend principally on the proportion of granules to fibrils. Complete inhibition of ATP splitting and contraction of the fibrils is achieved when the amount of nitrogen in the fibrils is 25 times as great as that in the granules.[52] Fifty per cent inhibition occurs when 1 mg. microsome nitrogen is mixed with 60 mg. fibril nitrogen[52] (FIGURE 3).

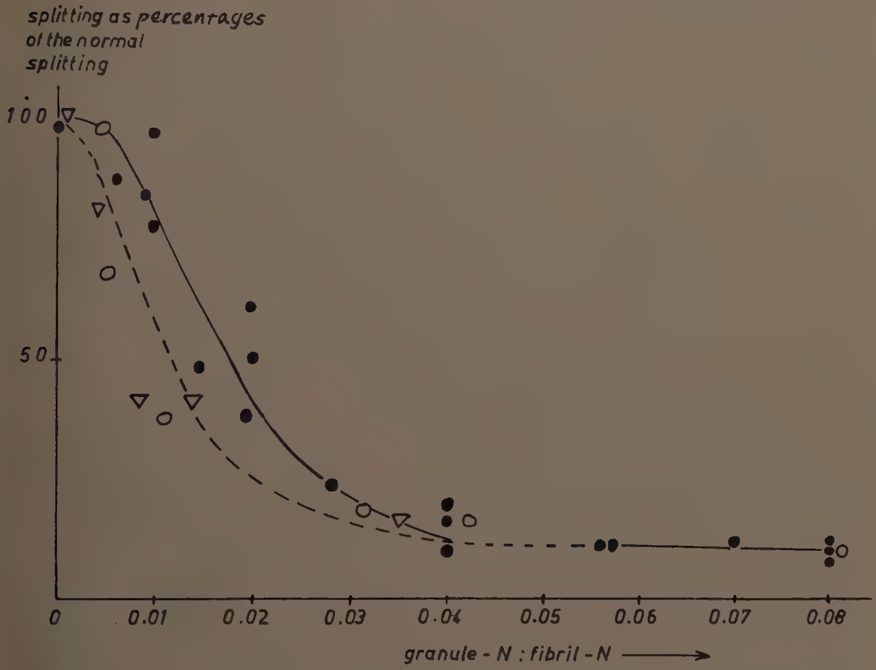

FIGURE 3. Dependence of ATP splitting on the proportion of granules to fibrils. Symbols: ●, 0.4 mg. protein/cc.; ○, 4 mg. protein/cc.; ▽, 9 mg. protein/cc. (according to Nagai *et al.*[52]).

(3) Nevertheless, a granule fraction can be washed in the centrifuge three times with a one hundredfold volume of ATP solution either in the absence or presence of ATP without the factor substance being given off to the solution and without the granules losing their relaxing effect (FIGURE 4).

(4) If, on the other hand, a greater amount of ATP and fibrils is added to the granule suspension and later centrifuged off at high speed, the suspension loses its relaxing effect, more or less (TABLE 1), without any decrease in the concentration of the granules estimated as turbidity (FIGURE 5).

All these considerations and observations permit the conclusion that the granules exchange a factor with the fibrils, but they do this only when in contact with the fibrils.

For the transition of the relaxing factor from the granules to the fibrils, ATP must be present as well. If granules are added to the ATP-containing fibrils in a quantity that produces only partial inhibition to ATP splitting,

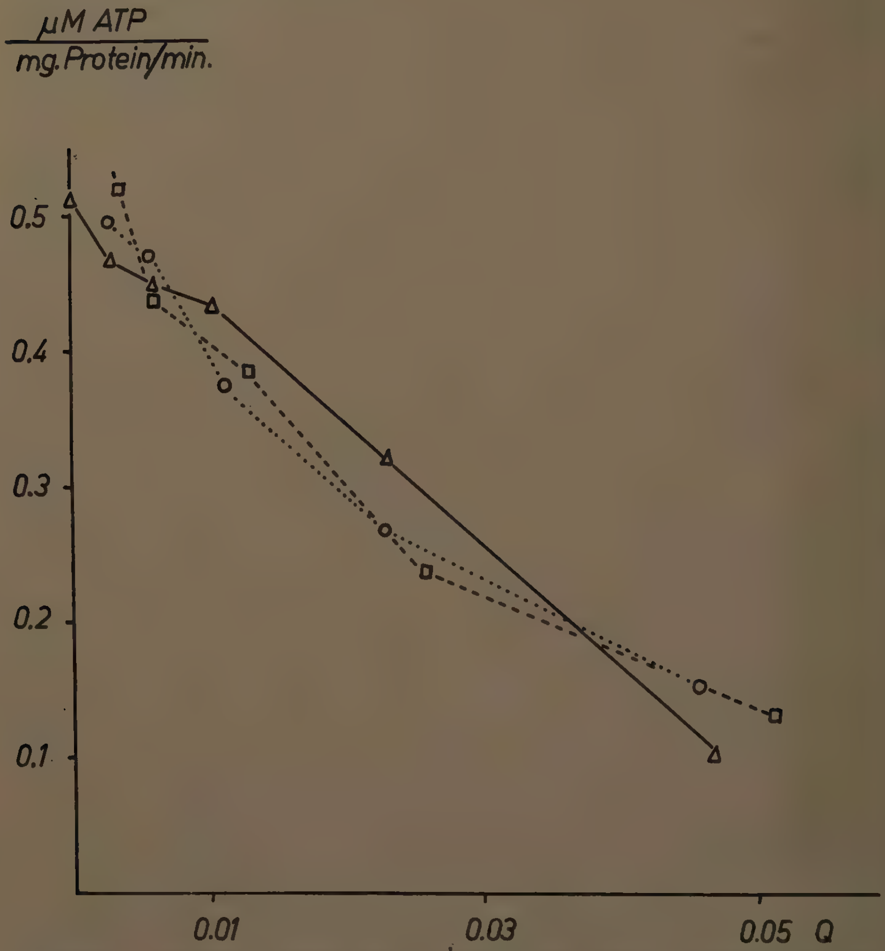

FIGURE 4. The effect of washing on the activity of the granules. Ordinate, ATP splitting; abscissa, Q = mg. granule N : mg. fibril N. Symbols: △——△, granules before washing; ○····○, granules washed 3 times with the one hundredfold volume of 0.1 M KCl + 5 × 10^{-3} M oxalate solution; □----□, granules washed in the same way, but in the presence of 5 × 10^{-3} M Mg + 5 × 10^{-3} M ATP (pH 7.0, T = 20° C.).

ATP splitting at pH 7.0 (histidine buffer), 5 × 10^{-3} M ATP, 5 × 10^{-3} M Mg, 0.1 μ, T = 20° C. (according to Nagai *et al.*[52]).

this splitting is inhibited during the first three minutes to a lesser degree than during the remaining time of the experiment. This period of latency of the granule effect cannot be shortened if the granules are incubated with the fibrils before adding the ATP. On the other hand, the inhibition is effective from the very start if the granules have been incubated with ATP before beginning the experiment (FIGURE 6). Of course, it is still an open question whether it is the emission or the production of the relaxing factor that the ATP makes possible or promotes by means of the granules.

TABLE 1
THE DECREASE IN THE GRANULE EFFECT CAUSED BY WASHING WITH A FIBRIL SUSPENSION[52]

Number of washings	Inhibition of ATP splitting* by the granules after washing (per cent†)	
	With fibrils‡	Without fibrils
1	20	44
1	76	100
2	4	100
1	100	100
2	12	97
1	94	97
2	64	94

* Granules: fibrils during splitting 1:30.
† 100% = Maximal inhibition produced by an excess of granules.
‡ One mg. of granules has been washed with 400 mg. fibrils once or twice.

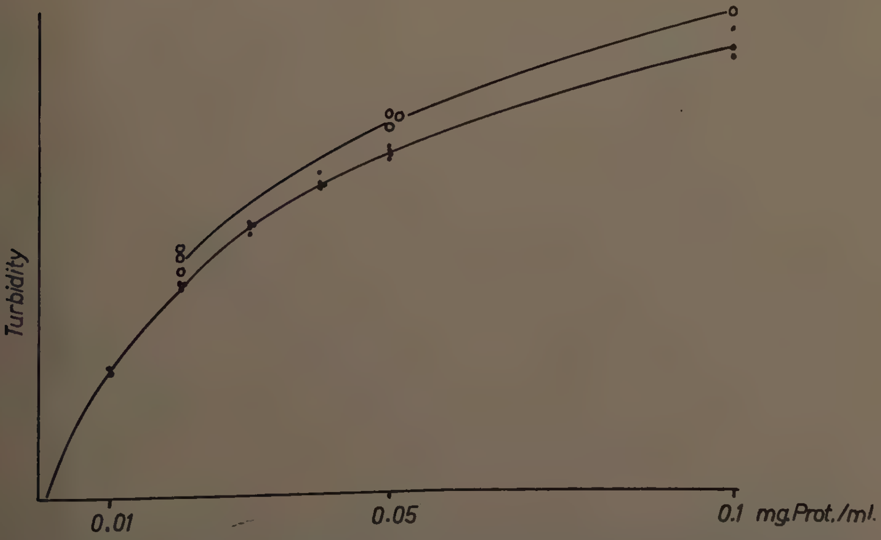

FIGURE 5. Turbidity of the granules before and after washing with fibrils. Symbols: ●——●, dependence of the turbidity on the concentration of granules before washing; ○——○, the same after washing with 20 mg./cu. cm. of fibrils (according to Nagai *et al.*[52]).

Because of the foregoing observations and conclusions, one expects that fibrils whose ATP splitting and contraction have been inhibited by granules afterward contain the relaxing factor in their interior. Consequently, such fibrils should not split ATP even if they are centrifuged off from the grana suspension and resuspended in pure ATP solution. We were quite surprised to observe that, after the removal of the grana, fibrils immediately split ATP again with maximal speed[52] (FIGURE 7).

This surprising finding could be explained on the basis of the discovery of a cofactor by Gergely and his colleagues, that is reported in this monograph and elsewhere.[53-55]

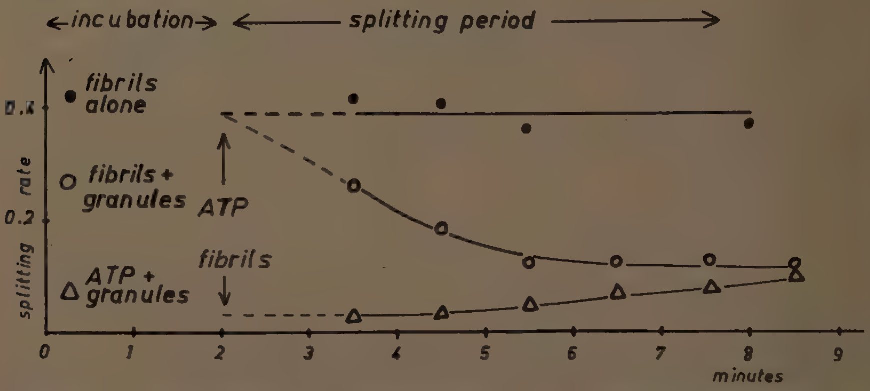

FIGURE 6. The influence of ATP on the activity of the ATPase-inhibiting granules (according to Nagai *et al.*[52]).

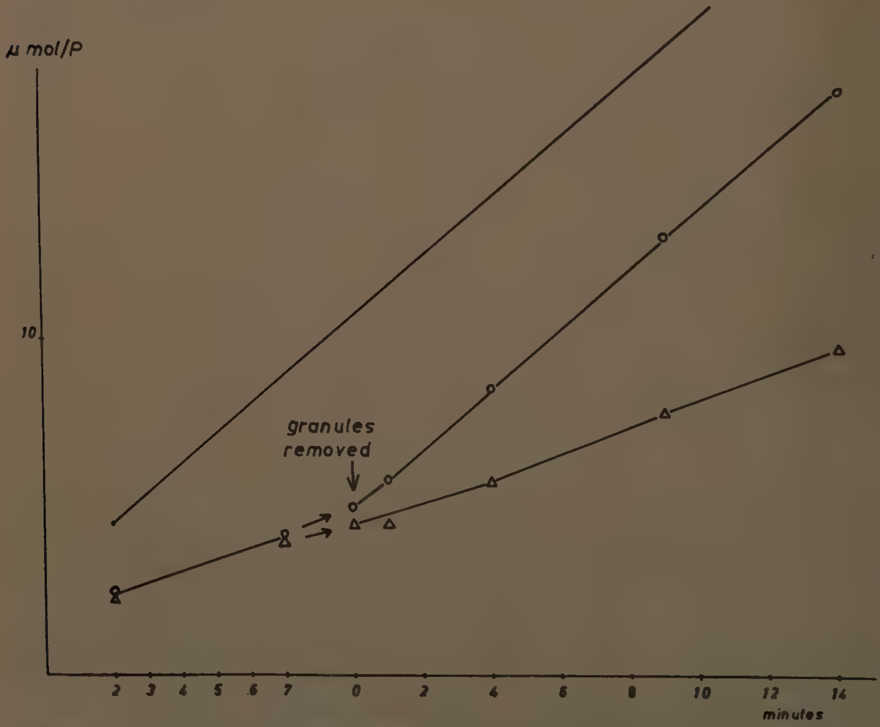

FIGURE 7. The influence of the cofactor on the lifetime of the granule factor. Ordinate, splitting rate of ATP in μmol/*P*; abscissa, time in minutes. Symbols: ●, uninfluenced fibrils; ○, fibrils + granules; △, fibrils + granules + cofactor (according to Nagai *et al.*[52]).

Briggs *et al.*[53-55] have discovered a cofactor that under certain conditions promotes to an extraordinary degree the relaxing effect of the granules. In the absence of granules, this cofactor seems to have no inhibiting effect. If, however, the granules are removed from the cofactor-containing mixture, the ATP splitting by the fibrils does not return to its normal value, but the inhibition of the splitting remains.[52]

In other words, if the granules are removed from a cofactor-free fibril suspension, the inhibition of ATP splitting is immediately terminated. However, if they are removed from a cofactor-containing solution, the inhibition of the splitting is retained. These two observations lead to the following conclusion: the granules give off a relaxing factor to the fibrils that is continuously and quickly destroyed by the fibrils. The cofactor, however, prevents such destruction. I think that this conclusion can explain certain observations made by Gergely and his colleagues in their presentation.

By reason of the fact that the physiological relaxing system of the muscle, as well as many poisons, puts an end to the contraction of the fibrils by inhibition, the splitting of ATP fits in with my theory concerning the sliding filaments. According to this theory, contraction is based on ATP splitting. However, in addition, the behavior of the physiological relaxing factor supports the theory in a special way. The fact is that complete relaxation is effected by the system of the physiological relaxing factor without completely inhibiting ATP splitting. It is even impossible to achieve by means of the relaxing factor a complete inhibition of ATP splitting.[52] FIGURE 3 shows that ATP splitting can be inhibited even in a factor excess of not more than 90 per cent of uninfluenced splitting. The remaining 10 per cent of the splitting, however, represents exactly the splitting rate that occurs when ATP is split by pure L-myosin. On the other hand, the ATPase activity of pure L-myosin is not influenced by the physiological relaxing factor (TABLE 2). This is the difference between the relaxing factor and the ATPase poisons. The relaxing factor, moreover, converts the highly effective Mg^{++}-activated actomyosin ATPase into Mg^{++}-activated L-myosin ATPase, which is only slightly effective; that is to say, the relaxing factor and the ATP put an end to the interaction between actin and L-myosin, on which interaction the high rate of ATP splitting in the actomyosin system is based.

TABLE 2
THE INFLUENCE OF THE RELAXING FACTOR ON ATP SPLITTING BY L-MYOSIN[52]

Proportion of granule N to L-myosin N	Splitting rate as μmoles P per mg. protein/min.
0	0.016
0.01	0.016
0.02	0.013
0.04	0.016
0.06	0.017

The observations in the mechanical properties of water-glycerin-extracted fibers also reveal that the relaxing factor in the presence of ATP terminates the association between actin and L-myosin. We know from the presentation of H. E. Huxley and J. Hanson that stretching of a fiber produces slipping of its filaments. Therefore, the degree of association of the filaments can be estimated from the resistance against stretch, that is, from the resistance against slipping. The resistance of water-glycerin-extracted fibers to stretch, however, becomes practically zero if factor and ATP are both present. When ATPase poisons are present, the resistance to stretch of the ATP-containing fibers decreases considerably because ATP splitting is inhibited, but remains considerably higher than in the presence of the relaxing factor[9, 48] (TABLE 3).

A highly convincing argument in support of the theory of sliding filaments is the fact that contraction ceases completely as soon as the interaction between actin and L-myosin has been stopped, and that part of the ATP splitting that does not depend on this interaction is irrelevant to contraction.

TABLE 3

STATIC RESISTANCE AGAINST STRETCH (EXTRACTED MUSCLE FIBERS)

	As gm. $\times$ cm.$^{-2}$ $\times$ L $\times$ ΔL^{-1}
Rigor	~10000
Contraction	~5000
Relaxation	
produced by Mersalyl	~250*
produced by factor	~0†

* Portzehl.[9]

† Hasselbach and Weber.[48]

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Part II. Excitation-Contraction Coupling

IMPULSE PROPAGATION IN STRIATED MUSCLE FIBERS AND THE ROLE OF THE INTERNAL CURRENTS IN ACTIVATION

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Whenever a nerve impulse arrives at the neuromuscular junction, the entire muscle fiber is activated almost simultaneously. This is brought about by the wave of excitation that travels rapidly along the fiber surface, in its passage stimulating the contractile substance. The study of excitation in muscle involves two aspects: (1) problems concerned with the conduction mechanism, and (2) problems dealing with the way in which the electric change at the surface activates the contractile material.

(1) *Conduction Velocity and the Refractory Period*

The present report deals chiefly with the conduction of excitation in skeletal muscle. In discussing the mechanism of this process in muscle, much of the knowledge that relates impulse conduction in nerve to changes in ionic permeability may be applied (for references see Hodgkin 1951, 1957). Electric methods are profitable for certain aspects of the problem and, in the material presented here, the problem is dealt with in terms of the core-conductor model. In this connection, the thorough analysis of passive electric properties of muscle by Katz (1948) has been of importance for the work of our group.

In electric terms, the wave of excitation consists of a brief change in membrane potential that moves along the muscle fiber. Excitation occurs at every point at which the resting potential of the surface membrane is displaced to a critical level. At this point there occurs in the membrane an instability that causes the further change in potential to continue by itself. The spread of activity is transmitted by local currents flowing between resting and excited regions of the fiber surface.

The time course of an intracellularly recorded action potential from a muscle fiber differs essentially from that of an unmyelinated nerve fiber (FIGURE 1), as in the squid giant axon (Hodgkin and Huxley, 1945). The initial phase of repolarization to about −70 mV occurs within 3 msec. (20° C.). However, unlike nerve, an additional 30 msec. is required for restitution of the resting membrane potential. This means that repolarization is still in progress when the peak of isometric twitch tension has been reached. There is no phase of hyperpolarization, as in the squid axon. The region of the fiber over which the spike component of the internal action potential is spread can be determined from the duration of the spike and the rate of its conduction. It is of the same order in the muscle fiber as in the squid giant axon (7 to 10 mm.).

Knowledge of the conduction of the action potential is pertinent to a discussion of excitation-contraction coupling; in relating electric and mechanical

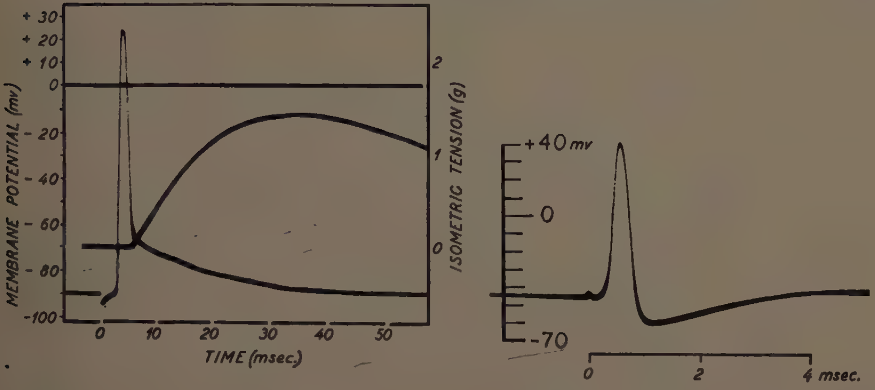

FIGURE 1. *Left:* internal action potential of a fiber and isometric twitch tension of the frog's m. extensor longus dig. IV (20° C.). *Right:* internal action potential of a squid giant axon (room temperature). From Hodgkin and Huxley, 1945; reproduced by permission from *The Journal of Physiology.*

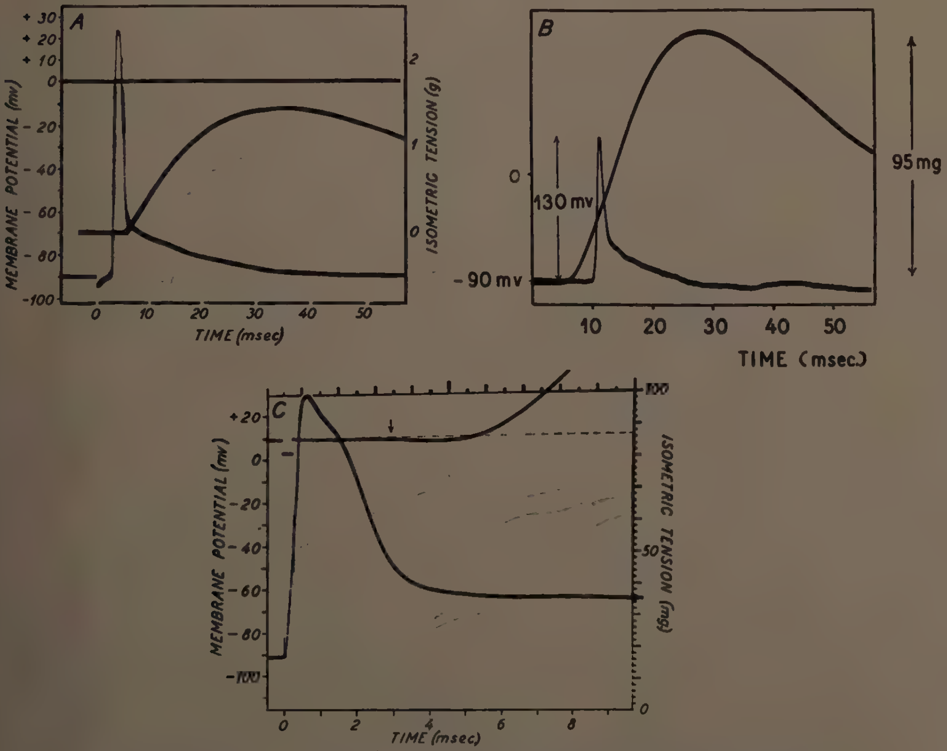

FIGURE 2. Relationship between action potential and isometric twitch tension (20° C.). *A*, intracellular fiber action potential, led off 3 mm. from the point of stimulation, and tension of whole m. extensor longus dig. IV; *B*, intracellular action potential and mechanical response of isolated fiber. The action potential was recorded at the fiber end opposite to that where stimulation was applied. Fiber length 20 mm.; *C*, intracellular action potential and mechanical response of whole m. extensor longus dig. IV all-over stimulated by a transverse pulse.

responses, conduction velocity obviously must be taken into account to avoid finding a contraction-excitation coupling. The influence of the conduction velocity on the recorded time relationship between the electric and mechanical

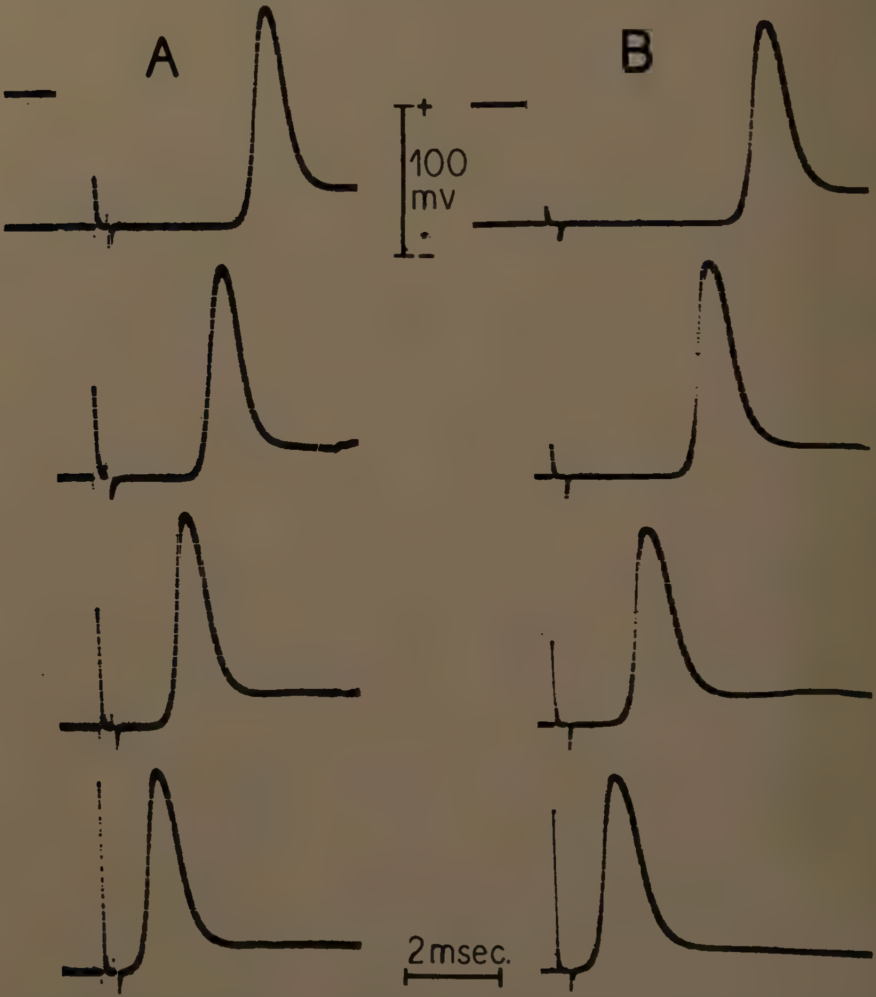

FIGURE 3. Two examples of conduction of the intracellularly recorded action potential in fibers from the frog's sartorius muscle. The potential was led off at 4 different distances from the intracellularly applied stimulating electrode (see FIGURE 4).

response is shown in FIGURE 2; *A* represents the isometric twitch tension of the curarized m. extensor longus digiti IV, and the action potential recorded from within one of its fibers. The electrode was placed about 3 mm. from the point of stimulation. If the intracellular electrode is placed near the tendon end opposite to the point of stimulation, as in FIGURE 2*B*, mechanical tension appears to precede the action potential.

A true picture of the time relationship between the wave of excitation and the mechanical response is shown in FIGURE 2*C*. The curarized foot muscle was stimulated by a transverse rectangular pulse similar to the mode of stimulation extensively used by Sandow (1948). The peak of the action potential was attained within 0.7 msec. (20° C.), independent of where the electrode was placed along the fiber (Guld and Sten-Knudsen, unpublished data). Thus, in this instance there is no longer any longitudinal conduction, conduction being confined to the transverse path around the 150 μ surface from the starting point of excitation on the fiber to the opposite side. This conduction lasts less than 0.1 msec. Probably this potential is closely related to the true active membrane potential changes associated with the ionic permeability changes and undistorted by the electrotonic effects accompanying longitudinal conduction. Mechanical force was recorded with high gain, so that latency relaxation could just be discriminated. The time interval between the peak of the action potential and the onset of latency relaxation was 2 msec. (20° C.), and the interval between the peak of the action potential and the onset of tension development was 4.3 msec.

The velocity of impulse conduction along the muscle fiber has been measured in two ways: (a) using intracellularly inserted electrodes for stimulation of and recording from single fibers *in situ*, and (b) using extracellular stimulation and recording both from the isolated fiber immersed in Ringer's solution and with the fiber *in situ*.

In both methods of determining conduction velocity it is important to measure the arrival time of the action potential at at least two points along the fiber (FIGURE 3). Plotting the arrival times as a function of the distance along the fiber extrapolation of the straight line between them to its intersection with the abscissa indicates the site at which the impulse is initiated (FIGURE 4). This is frequently situated at a distance from the stimulating electrode. Furthermore, the position of the true point of stimulation may vary with the intensity of the stimulus. In experiments where more than one fiber is activated by extracellular stimulation it is even necessary to record from at least 3 points of the fibers in order to ascertain that the potentials recorded originate from the same fibers as indicated by a rectilinear relationship between arrival time and distance.

Stimulating and recording intracellularly the rate of conduction in surface fibers of whole frog muscle averaged 2.3 ± 0.05 m./sec. (corrected to 20° C.) with a range between 1.8 and 2.9 m./sec. (Buchthal and Engbaek, 1957).

It seemed natural to attribute the variation in propagation velocity to the differences in fiber diameter that spread between 50 and 135 μ. However, a correlation between rate of conduction and fiber diameter could not be obtained with sufficient accuracy in whole muscle.

In isolated fibers the diameter can be determined accurately. Håkansson (1956) stimulated and recorded extracellularly from isolated frog muscle fibers immersed in Ringer's solution. The average rate of conduction was about the same as in surface fibers of whole muscle. Furthermore, there was a proportionality between fiber diameter and rate of conduction (FIGURE 5). Measurements of velocity on intrafusal fibers of muscle spindles which have an average diameter of 15 μ and a conduction rate of 0.3 m./sec. fit this rectilinear relationship (Buchthal and Jahn, 1957).

A rectilinear relationship between fiber diameter and rate of conduction is at variance with the square root relationship to be expected from core-conductor theory, assuming the same specific membrane capacity and resistance for thin and thick fibers. This is seen from the expression describing rate of conduction (V) in terms of specific membrane resistance (R_m) and capacity (C_m), specific internal resistance (R_i), fiber diameter (D), and safety factor (S) (Katz, 1948):

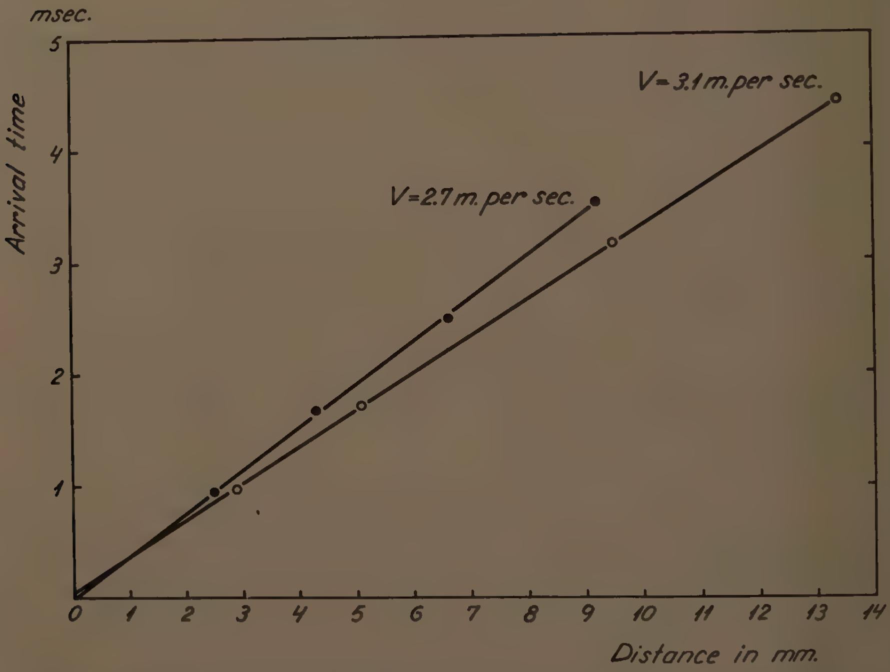

FIGURE 4. Arrival time of the action potentials at 4 different points along the fiber as a function of the distance from the stimulating electrode; same fibers as in FIGURE 3. V = 2.7 m./sec. (24.6° C.); V = 3.1 m./sec. (21.6° C.).

$$V = \frac{S}{2\sqrt{R_i}} \cdot \frac{\sqrt{D}}{C_m\sqrt{R_m}} \tag{1}$$

In this expression the external resistance is disregarded.

A different specific membrane capacity and resistance in thin and thick fibers could explain the proportionality between velocity and diameter. Systematic measurements of these properties in fibers of different diameter are not available. Assuming that membrane resistance varies in inverse proportion to fiber diameter and that membrane capacity remains constant, a rectilinear relationship would exist between rate of conduction and fiber diameter. However, this interpretation does not explain the fact that the velocities of intrafusal fibers fit the same relationship as found for twitch fibers. Koketsu and Nishi (1957) found specific membrane resistance and capacity to be about the same in the thin intrafusal fibers as in twitch fibers. If the core-conductor model is applicable at all, it would imply a smaller safety factor in intrafusal fibers than in twitch fibers.

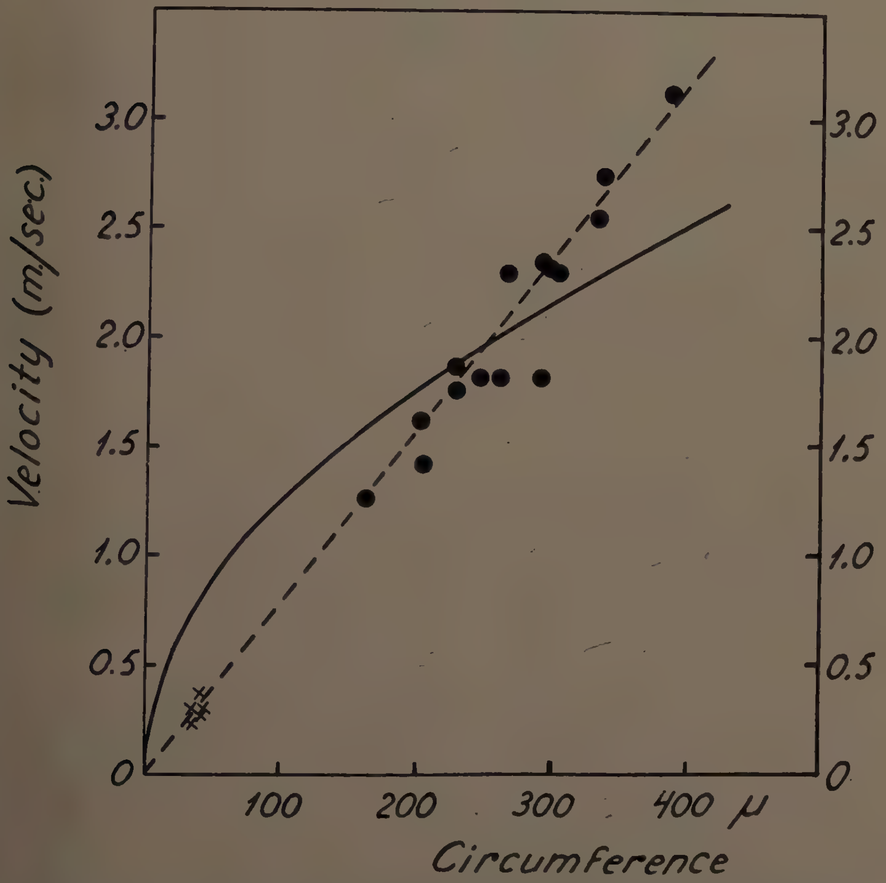

FIGURE 5. Conduction velocity of the action potential as a function of circumference in isolated frog muscle fibers immersed in Ringer's solution (20° C.). Symbols: (●) extrafusal twitch fibers (Håkansson, 1956); (×) intrafusal fibers (Buchthal and Jahn, 1957). Full line represents the square-root relationship to be expected from core-conductor theory.

In agreement with what has been described in nerve fibers (Hodgkin, 1939), conduction rate decreases with an increase in external resistance. Thus, the conduction rate over the muscle fiber surrounded by a thin Ringer's film was only one half that when the fiber was immersed in a large volume of Ringer's solution (Håkansson, 1957*a*). High external resistance has another effect that has not attracted attention; it alters the relationship between conduction rate and fiber diameter. In deep fibers *in situ* in mammalian muscle, velocities varied only by ±15 per cent, in spite of a threefold variation in fiber diameter. This behavior can be expected from the core-conductor model if the external longitudinal resistance per centimeter is similar to the internal resistance. That this is the case could be deduced from the values of specific membrane resistance and capacitance of the fiber and from the extent of the extracellular space (Buchthal *et al.*, 1955).

With the rectilinear relationship found in Ringer's solution between fiber diameter and conduction velocity, and taking into account the effect of external resistance, which was disregarded in EQUATION 1, conduction rate (V) varies with diameter (D) as

$$V = a \frac{D}{\sqrt{1 + \frac{r_e}{r_i}}} \tag{2}$$

with r_e the external longitudinal resistance per centimeter, r_i the internal longitudinal resistance per centimeter, and a a proportionality factor. Assuming a constant external resistance for fibers of different diameter, the ratio between external and internal resistance varies proportionally with the square of the diameter, hence

$$V = \frac{aD}{\sqrt{1 + kD^2}} \tag{3}$$

where k is a factor independent of D. This relationship is plotted for different values of k in FIGURE 6. With $k = 10^5$ cm.$^{-2}$, the conduction rate varies merely ±16 per cent over the range of diameters of 30 to 90 μ. This value of k corresponds to a longitudinal external resistance of $29 \cdot 10^6\ \Omega$/cm. and is 3.6 times the internal resistance of a 60-μ fiber.

A word about our findings on single fibers as compared with findings in whole muscle. In whole frog's muscle, conduction rate has been determined from the distance between the two phases of the diphasic compound action potential. This method gives an average velocity, and the range of velocities cannot be determined with accuracy. Eccles *et al.* (1941) and Katz (1948) have found average velocities in whole sartorius and extensor digitorum quartus of 1.6 to 1.8 m./sec., with a range between 1.0 to 2.2 m./sec. Martin (1954) found a slightly higher average velocity (1.9–2.3 m./sec.), which he explains by selection of especially large-sized frogs with a higher average muscle fiber diameter. Thus, conduction rate in isolated fibers in Ringer's solution and in single surface fibers of whole muscle (2.3 m./sec.) exceeds that in whole muscle by about 30 per cent. Furthermore, the range of velocities found in a population of isolated fibers is larger than that in whole muscle (1.0 to 3.1 m./sec., as compared to 1.0 to 2 m./sec.). The cause of this difference is twofold: first, the higher external resistance in whole muscle reduces the conduction rate; second, with more than one active fiber, interaction between adjacent fibers forces them to propagate their potentials synchronously, resulting in an equalization of conduction rate and hence a narrowing of the range of velocities (Buchthal *et al.*, 1955). This interaction has previously been demonstrated in nerve

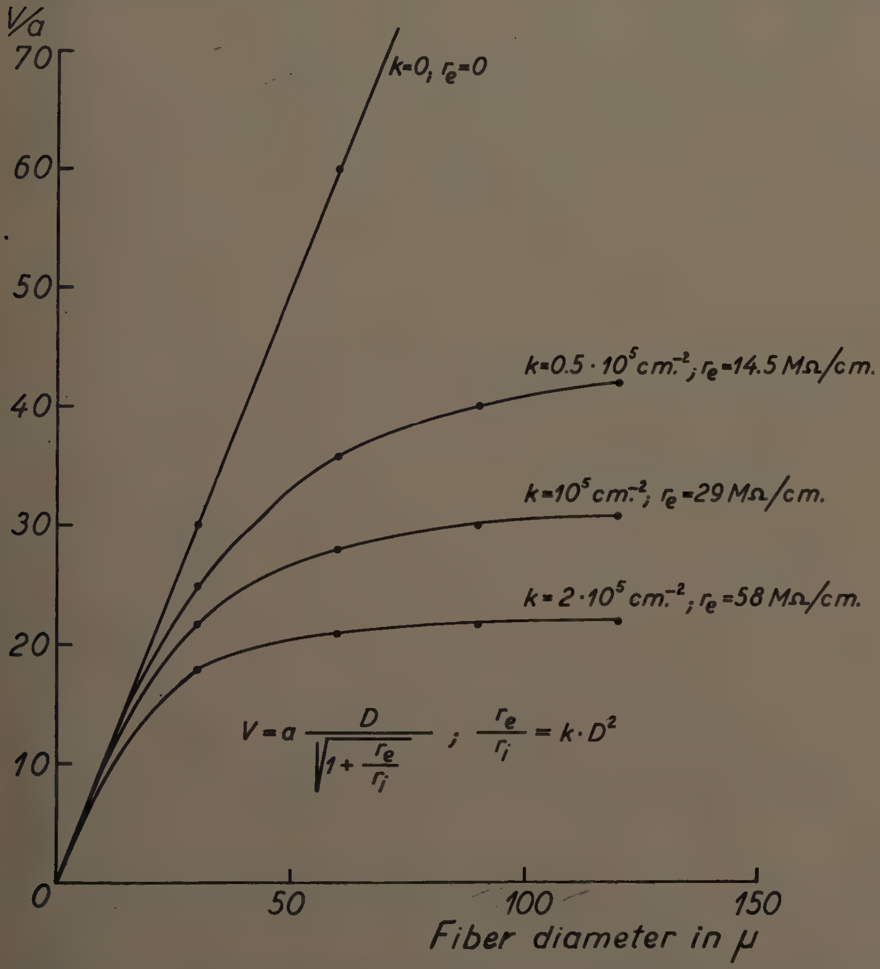

FIGURE 6. Influence of the external resistance (r_e) on the relationship between conduction velocity (V) and fiber diameter (D). With the fiber in Ringer's solution ($r_e = 0$) proportionality is assumed between velocity and diameter (see FIGURE 5).

fibers (Katz and Schmitt, 1940). That such interaction between adjacent muscle fibers actually occurs is indicated by the smooth course of the steep deflection of the motor unit potential, which lasts merely 100 to 200 μsec., although the spikes have been shown to arise from 10 to 30 muscle fibers (Buchthal *et al.*, 1957).

There are other conditions that alter conduction velocity: change in the potassium concentration of the surrounding medium, temperature, stretch, denervation, and a preceding action potential.

A fivefold increase in external potassium depolarizes the fiber to such a degree that it no longer conducts. A threefold increase in potassium concentration (6 mM) that caused a decrease in the membrane potential and in the action potential of 15 per cent was associated with a 20 per cent decrease in rate of conduction (FIGURE 7, Buchthal and Engbaek, unpublished data).

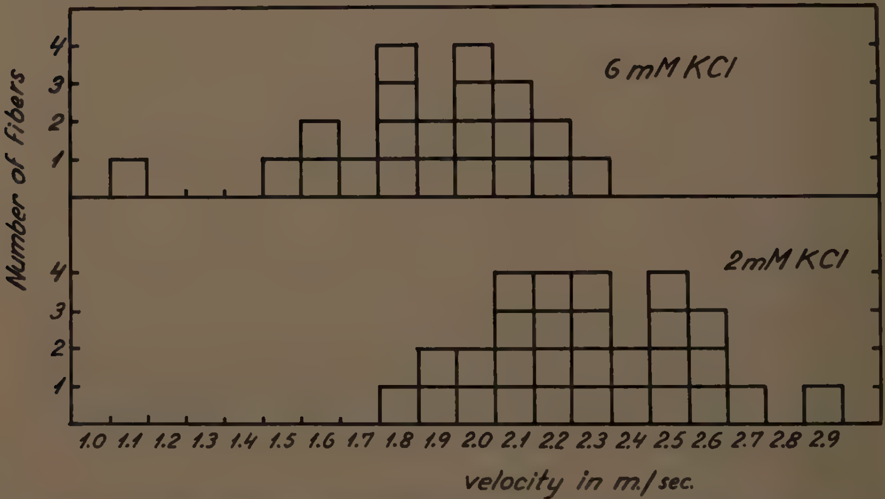

FIGURE 7. Distribution of intracellularly recorded conduction velocities in fibers of the frog's sartorius muscle with normal (2 mM) and increased (6 mM) external potassium concentration (corrected to 20° C.). See Buchthal and Engbaek, 1957.

The specific membrane resistance of a muscle fiber immersed in Ringer's solution containing 6 mM potassium chloride was 40 per cent less than when the Ringer's solution contained 2 mM potassium chloride (Jenerick, 1953). In terms of the core-conductor model, this causes a decrease in the characteristic length of 20 per cent; disregarding possible changes in the safety factor, the rate of conduction would thereby be reduced by 20 per cent.

A decrease in temperature slows conduction velocity, the temperature coefficient (Q_{10}) being about 2 between 10 and 20° C. in whole frog's muscle (Eccles *et al.*, 1941) and in isolated muscle fibers (Håkansson, 1954). With a further decrease in temperature, the temperature coefficient decreases. Similarly, in fibers from intact human muscle the temperature coefficient (Q_{10}) was 2 in the range of 30 to 37° C., and between 26 and 30° C. there was no significant change in conduction velocity with temperature (Buchthal and Rosenfalck, unpublished data).

Muscle fibers are highly elastic, their diameters being substantially reduced by stretch. The question then arises whether a change in diameter induced by stretch influences rate of conduction as expected in view of the proportionality between fiber diameter and conduction rate in different isolated fibers at the same degree of stretch. This is not the case. Martin (1954) found that stretch of whole muscle did not alter conduction rate up to 50 per cent of stretch above equilibrium length.* Similarly, conduction velocity in isolated fibers immersed in Ringer's solution remained unaltered up to 50 per cent stretch. Isolated fibers can be extended to a greater degree than whole muscle and, at elongations above 50 per cent of equilibrium length, rate of conduction increased in spite of the reduction in diameter (Håkansson, 1957*b*). This effect is fully reversible and most pronounced in fibers of large diameter whose rate of conduction increased 50 per cent with a decrease in diameter of 30 per cent (FIGURE 8).

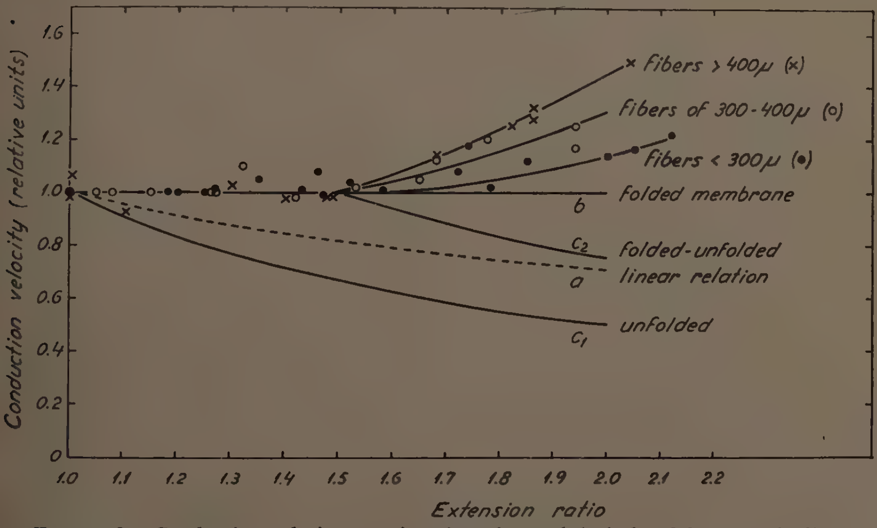

FIGURE 8. Conduction velocity as a function of stretch in isolated frog muscle fibers of different circumference immersed in Ringer's solution (20° C.): *a*, calculated assuming proportionality between fiber diameter and conduction velocity; *b*, c_1, and c_2 calculated from the folded membrane model. See Håkansson, 1957*b*.

To account for the constant velocity over a large range of stretch, it has been suggested that the muscle fiber membrane is "folded" (Katz, 1949; Martin, 1954); a folded membrane would account as well for the membrane capacity of muscle fibers fourfold greater than that of nerve. Assuming the membrane to be unfolded at degrees of stretch greater than 50 per cent, a further elongation would be associated with a decrease in propagation velocity, assuming that membrane resistance decreases proportionally, and membrane capacity inversely proportionally, to the thickness of the membrane (Martin, 1954). Since velocity actually increases at degrees of stretch greater than 50 per cent, the passive electric properties of the unfolded membrane do not behave during stretch as assumed by Martin (1954). Since, in addition, the threshold did not decrease, probably both specific membrane capacity and resistance decrease with stretch.

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* Martin's 67 to 120 per cent of resting length corresponds approximately to equilibrium length to 50 per cent stretch.

A reduction in muscle fiber diameter is also produced by denervation. In totally denervated human muscle there was no correlation between conduction velocity and reduction in fiber diameter: normal velocities were found in muscles with average fiber diameters one half those of normal muscle; velocities one third of the normal and less occurred in nonfibrillating fibers of denervated muscles in which the reduction in fiber diameter was only slight (FIGURE 9; see Buchthal and Rosenfalck, 1958), nor was the electric excitability of these fibers increased. To account for the decrease in conduction velocity, one must therefore assume a change in membrane properties, an increase in both specific membrane capacitance and resistance. In denervated frog's muscle, where rate of conduction was reduced by merely 10 per cent, Nicholls (1956) found a doubling of membrane resistance and a decrease in capacitance of 25 per cent.

It has been suggested that a high internal resistance accounts for a decrease in conduction velocity in denervated muscle fibers (Jarcho *et al.*, 1954). This can hardly be the case, however, since changes in internal resistance with the fiber *in situ* are reflected in changes of conduction velocity to only a minor degree (EQUATION 2).

The effect of a preceding (conditioning) action potential on the excitability of the membrane was investigated by intracellular recording at a distance of 5 mm. and less than 0.5 mm. from an intracellular stimulating electrode (Buchthal and Engbaek, unpublished data).

Leading off within 0.5 mm. from the point of stimulation (FIGURE 10), the fibers did not respond to a subsequent stimulus delivered 1.6 ± 0.1 msec. (25° C.) after the first stimulus. If the time interval was increased by about 0.3 msec., the testing stimulus evoked a nonpropagated response with a time course allowing it to be discriminated with certainty from the electrotonic response of the membrane.

For the second action potential to be propagated, an additional 0.2 msec. was required between stimuli, or about 2 msec. in all (25° C.). A local response occurred when the testing stimulus was applied about at the onset of the protracted phase of repolarization of the conditioning potential. There was no significant change in repolarization from the time at which a local response could be elicited to the minimum time required to obtain a propagated response. Hence, these excitability changes cannot be described in terms of changes in membrane potential.

At the end of the absolute refractory period, the time interval between stimuli was shorter than the time interval between the peaks of the corresponding propagated action potentials. At a distance between stimulating and recording

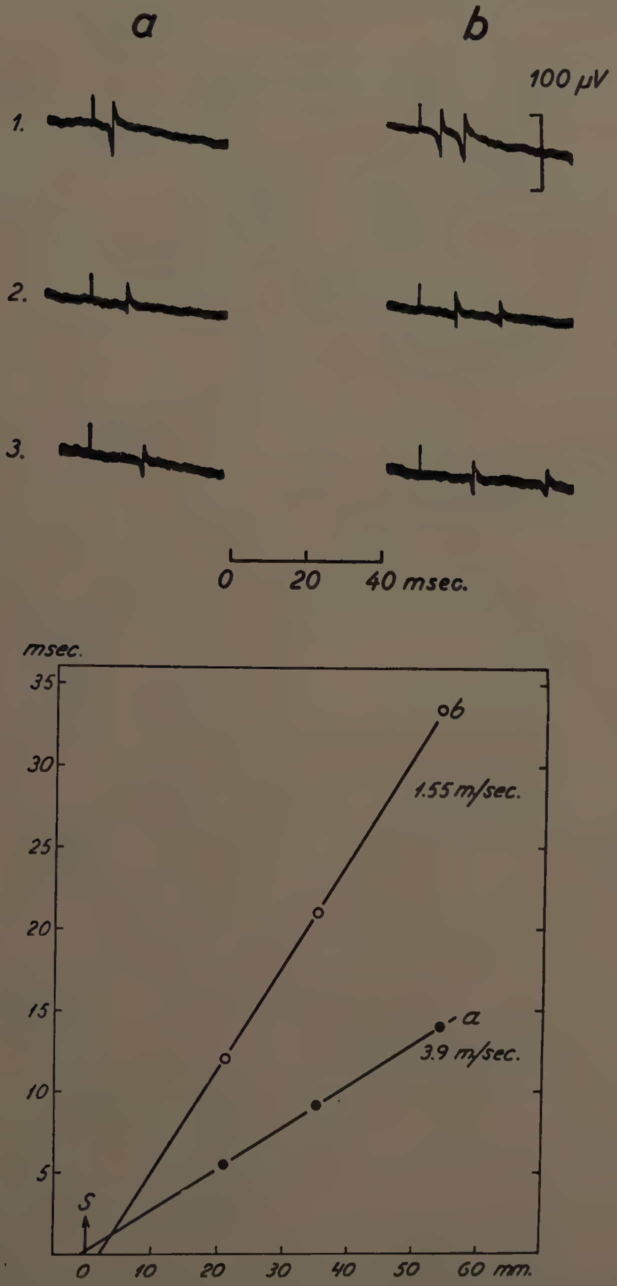

FIGURE 9. *Above*, action potential recorded at increasing distance from the stimulating electrode in a denervated human brachial biceps (36.5° C.): *a*, fiber with normal rate of conduction; *b*, in addition to *a*, a fiber with decreased rate of conduction. *Below*, arrival time of the action potentials as a function of the distance between stimulating electrode (*S*) and recording electrodes. From Buchthal and Rosenfalck, 1958; reproduced by permission from *Electroencephalography and Clinical Neurophysiology*.

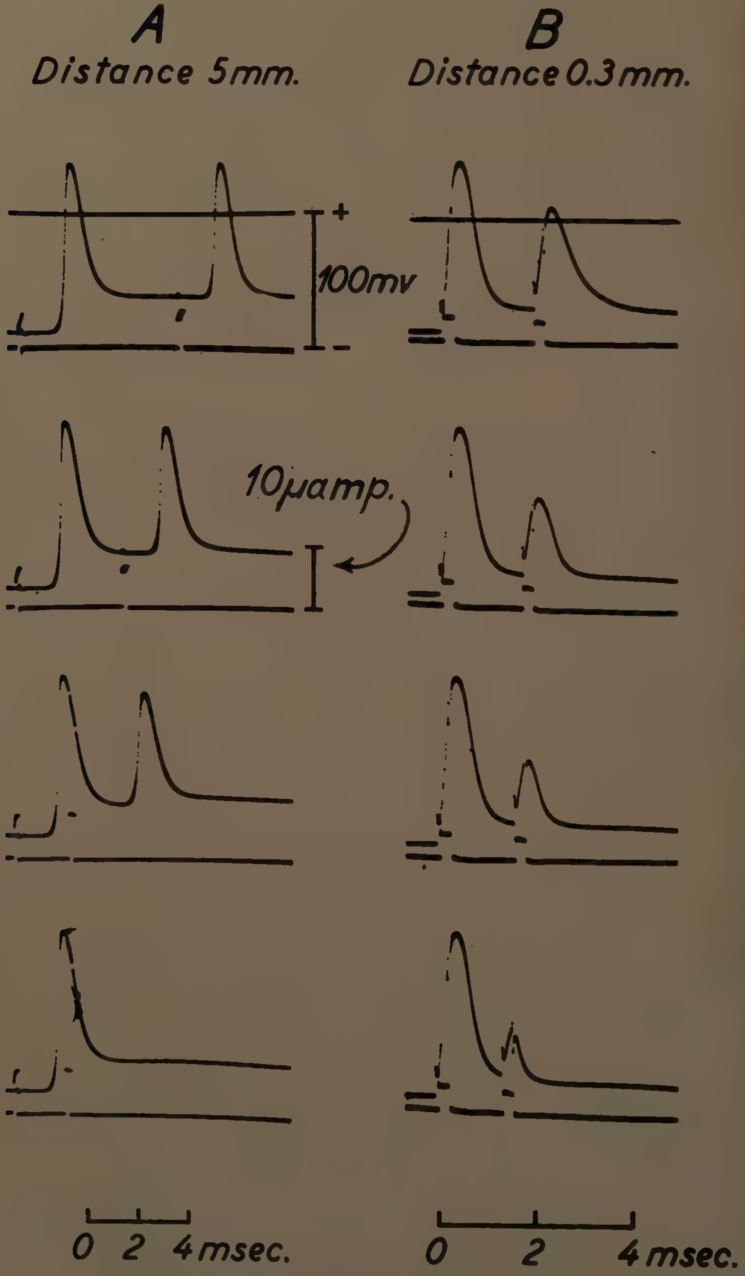

FIGURE 10. *Upper beam*, refractory period of the propagated action potential (*A*) and of the local response (*B*); intracellular stimulation and recording from fibers of the frog's sartorius muscle; distance between stimulating electrode and recording electrode 5.1 mm. (*A*, 23° C.) and 0.3 mm. (*B*, 24.5° C.). Time interval between conditioning and testing stimulus decreases from above to below. *Lower beam*, stimulating current.

electrode of 5 mm., the difference was 1 msec. (25° C.). Only to a minor degree was this difference due to a delay in the onset of the second action potential. A slowing of conduction of the second potential of about 35 per cent was the principal cause of the difference in time between the 2 stimuli and the 2 peaks of the action potentials.

These effects were not attributable to the degree of repolarization, as indicated by the fact that the 2 action potentials arrived after identical delays when the 2 stimuli were separated by 5 msec. At 5 msec. the negative afterpotential of the conditioning action potential is still at an approximately constant level of depolarization of 15 to 20 mV.

(2) *The Possible Role of Internal Longitudinal Currents in Excitation-Contraction Coupling*

The first step in the process initiating the mechanical response in a muscle fiber is the depolarization of the surface membrane of the fiber, as manifested by the propagated action potential. During the passage of an action potential a considerable current flows longitudinally in the interior of the muscle fiber; this amounts to 9 mAmp./sq. cm. during the maximum rate of rise of the action potential. It has been considered that these currents might stimulate the contractile material directly and thus represent the link between excitation and mechanical response (Bay *et al.*, 1953). Since, however, muscles stimulated electrically in the absence of the propagated action potential contract only at those points where the current leaves the fiber, that is, where the membrane is depolarized (Sten-Knudsen, 1954; Huxley and Taylor, 1955), this possibility has been discarded. There was no evidence of contraction in regions of the fiber where internal current flow is not associated with a depolarization of the membrane (Watanabe and Avabe, 1956).

Recently Csapo and Suzuki (1958) have revived the hypothesis that longitudinal internal currents are the immediate agents for initiating the process of contraction. They suggest that activation occurs in 2 steps: depolarization of the surface membrane was considered to be a priming step, and the internal longitudinal currents were assumed to activate the contractile material. The experimental evidence for this assumption is based on two observations:

First, application of a strong longitudinal alternating electric field (8 to 20 V/cm. at 60 cps) activates a muscle depolarized to about 50 mV by excess potassium in the surrounding fluid. Shortening was most pronounced in the middle portion of the muscle, and at threshold only this region shortened, the ends either shortening very little or not at all.

Second, when a muscle was suddenly exposed to contracture-producing amounts of potassium (more than 16 mM), mechanical tension was less than the tension produced in a normal tetanus.

Finally, in a normally propagating sartorius muscle activated by 60 cps, shortening was likewise most pronounced in the middle portion of the muscle.

In the whole sartorius muscle, fiber length and distribution are such that it seems hardly possible that all fibers can be aligned parallel to the direction of a homogeneous electric field. With the high field strengths applied by Csapo and Suzuki (1958) even a very slight misalignment results in undesired transverse effects of the currents.

Sten-Knudsen (unpublished data) has reinvestigated the effect of a longitudinal AC field using isolated fibers carefully aligned in the direction of the AC field. The fibers were depolarized by 14 mM potassium, as in Csapo and Suzuki's (1958) experiments. The changes in length of the different fiber portions were measured by recording the position of 20 to 30 small graphite granules distributed along the length of the fiber. An experiment of this type is illustrated in FIGURE 11. With a field strength of 0.73 V/cm., shortening was barely visible at the most terminal portions of the fiber. Increasing field strengths caused increasing shortening of both terminal portions; with a field strength of 5.55 V/cm., shortening of the fiber portion near the tendon end was almost 80 per cent. The shortened regions did not extend over more than 0.5 mm. at each tendon end of the 20-mm.-long fiber. Up to a field strength of 5.5 V/cm. applied for 2 sec., the contraction induced by a longitudinal field was fully reversible. The results of these experiments are at variance with Csapo and Suzuki's findings on whole muscle.

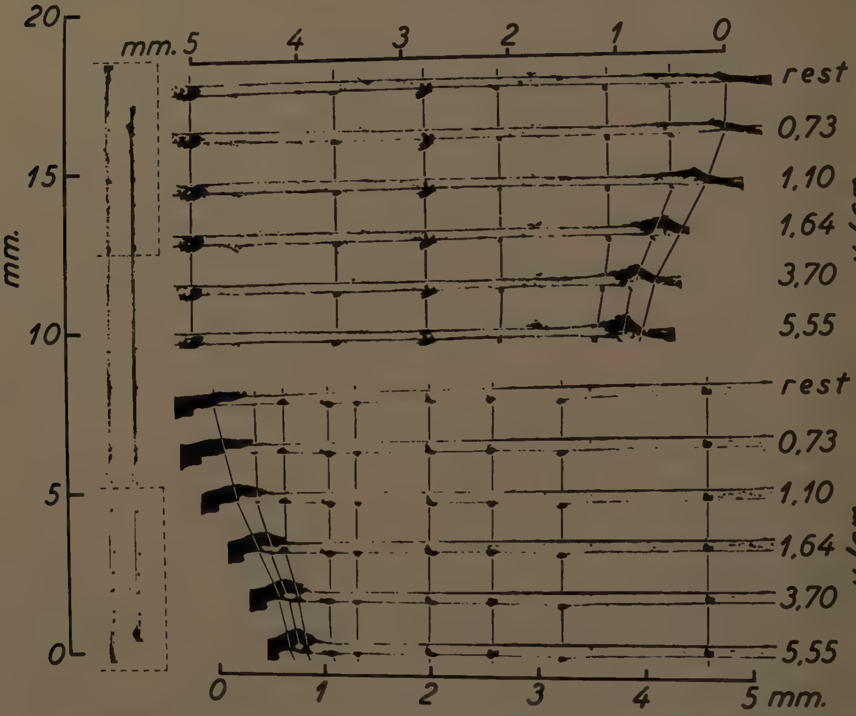

FIGURE 11. Isotonic shortening of a nonpropagating isolated muscle fiber (blocked with 14 mM KCl Ringer's solution) stimulated by a homogeneous longitudinal AC field at 100 cps; load on fiber 12 dynes, length at rest 17 mm. (20° C.). *Left*, fiber in its whole length (marked with graphite granules at rest and during stimulation—3.70 V/cm.); *above*, magnified lever end of the fiber at rest and during application of increasing field strengths; *below*, fixed end of the same fiber.

At field strengths exceeding 5.5 V/cm. the changes in length evoked by the longitudinal field were no longer reversible. At 5.8 V/cm., for example

(FIGURE 12), shortening was at first confined to the two ends of the fiber. After one second of stimulation, the current caused repeated slow waves of contraction, associated with local bulges and followed by a phase of relaxation. This behavior corresponds to the galvanic waves described by Hermann (1886), which he explained by assuming that local anodes form behind a bulge and local cathodes before it. After 3 sec. of current flow the fiber was shortened a few millimeters in all, but was still extremely shortened at both ends. With a large field strength of 7.3 V/cm. the galvanic waves occurred more frequently and were propagated over the whole length of the fiber, which ended up with a total shortening of 40 per cent. The use of isolated fibers allows microscopic observation of this effect, which is not possible when whole muscle is used. The fact that shortening induced by these field strengths is only partly reversible is illustrated in FIGURE 12, recorded 4 sec. after removal of a 3-sec. stimulation, in which both fiber ends remain shortened. The behavior was the same when 60 and 500 cps stimulations were applied and when depolarization was effected by 11 and by 14 mM potassium Ringer's solution, although the field strengths required were higher at the high frequency and at the low potassium concentration.

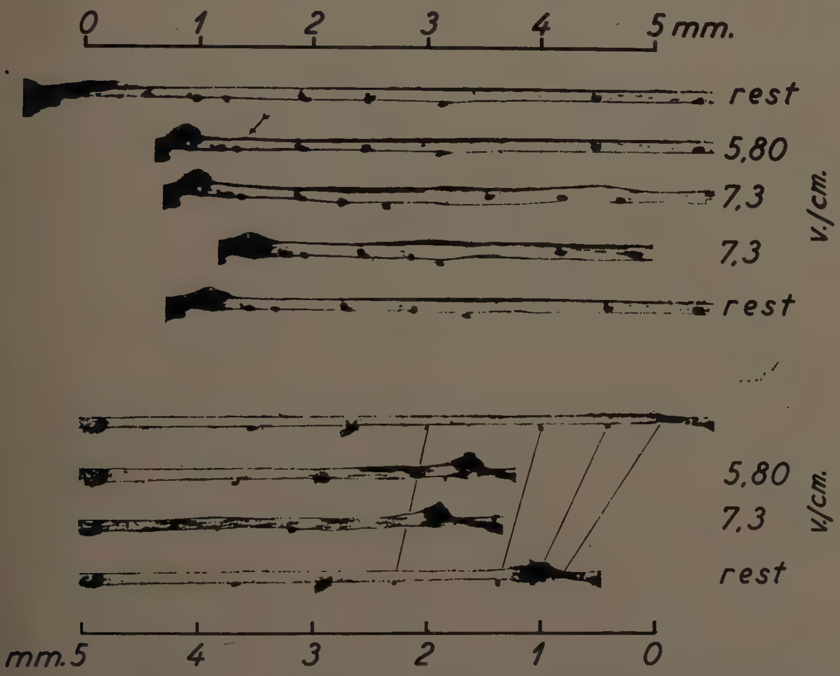

FIGURE 12. Same fiber as in FIGURE 11, showing the irreversible effect of supramaximal field strengths. Note the local bulges (*arrow*), and the irregular diameter and shortening during contraction. *Above,* fixed end; *below,* lever end.

Thus, an over-all contraction can be elicited only by excessively large field strengths, and the shortening produced is by no means uniform along the length of the fiber.

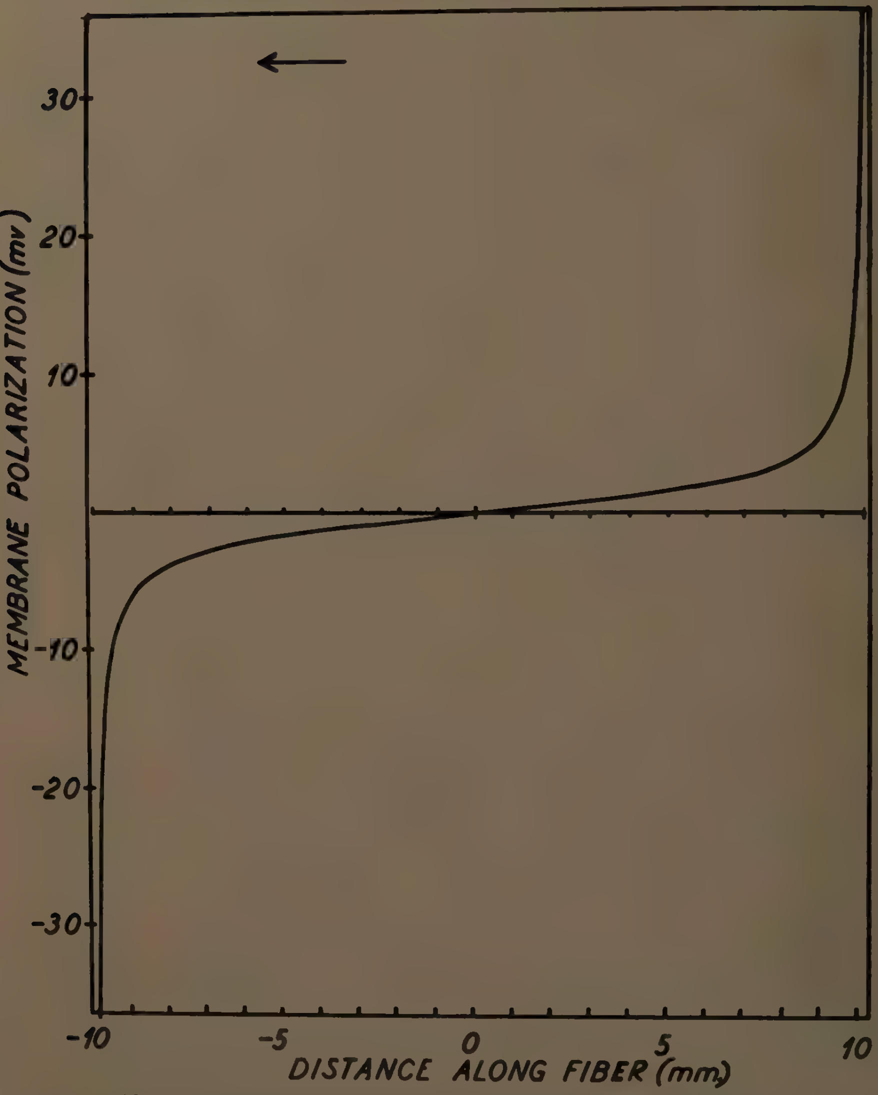

FIGURE 13. Membrane polarization along a 20-mm.-long muscle fiber stimulated by an AC field of 1 V/cm., calculated at the peak of a half cycle at 100 cps. Arrow shows direction of longitudinal field $E = 1$ V/cm.; V_m = membrane polarization along fiber surface;

$$-V_m = \frac{Z_m g_e[1 - Q_1(u_0)/u_0 Q_1'(u_0)]}{1 - Z_m g_1/f u_0 - g_1 Q_1(u_0)/g_e u_0 Q_1(u_0)} \left(\frac{u_0^2 - 1}{u_0^2 - v^2}\right)^{1/2} \times vE$$

Z_m = specific surface resistance = 400 ohm/sq. cm. at 100 cps; g_e = specific conductance of Ringer's solution = 10^{-2} mho/cm.; g_1 = specific internal conductance of fiber = $\frac{1}{300}$ mho/cm.; a = fiber length = 2 cm.; b = fiber diameter = 100 μ; u_0 = cosh [artanh (b/a)] = ellipsoidal coordinate defining fiber surface; $v = z/a$, where z = distance along fiber length, measured from origin; $Q_1(u)$ = Legendre function of second kind and first order; $Q_1'(u)$ = derivative of $Q_1(u)$; f = focal distance = a/u_0.

That an over-all shortening may be produced by a large field strength in a strongly depolarized fiber can be deduced from the course of the membrane polarization along the fiber as it occurs at the peak of a one-half cycle at 100 cps. By far the largest part of polarization occurs at the fiber ends, and the remaining fiber is subjected to a slow change in polarization (FIGURE 13). With the fiber initially depolarized almost to the contracture level by the high potassium and with large fields, the critical depolarization is exceeded over a considerable part of the fiber.

Provided that most of the membrane current is carried via the membrane capacity, a longitudinal field of 10 V/cm. at 60 cps, as applied by Csapo and Suzuki (1958), causes a maximal displacement of the membrane potential of several volts in 20-mm.-long fibers. Such polarizing voltages are outside the physiological range, and the irreversible shortening of the fiber ends may be the result of this excessive stimulation. That the middle portion of the muscle appears more shortened than the ends by large field strengths may thus result from a reduced response of the fiber ends damaged by preceding stimuli.

With respect to the second argument, namely, that less tension develops in potassium contracture than in normal tetanus, indicating that depolarization is less effective in eliciting a mechanical response than longitudinal current, the diffusion time for potassium to penetrate to the different fibers cannot be disregarded when a muscle is suddenly exposed to excess potassium.

The assumption that membrane depolarization is merely a necessary priming step for the effect of internal longitudinal current is difficult to prove or disprove. As outlined, Csapo and Suzuki's evidence does not seem convincing. Furthermore, if the assumption were correct, transverse internal currents probably should be just as effective as longitudinal ones in producing a response, since Sten-Knudsen (1954, 1957) has shown that nearly the whole tetanic tension can be produced by a transverse AC field applied to a nonpropagating muscle. Internal transverse current flow and threshold of the muscle response for frequencies of 200 to 5000 cps are shown in FIGURE 14. The threshold increases in spite of the increase in transverse current with increasing frequency. The opposite should be expected if transverse current flow were directly linked to the activation process. On the other hand, the frequency dependence of the threshold can be understood when the depolarization of the membrane is taken into account, determined solely by the reactance of the membrane capacity in that frequency range.

Volume Conduction of Muscle Action Potentials

In discussing conduction velocity we have mentioned internal and external recordings without accounting for the relationship between the intracellularly recorded and the volume-conducted potential. Any such comparison must take into account the resistance and the extent of the surrounding medium.

When the resistance of the surrounding medium is high as compared to the internal resistance of the fiber, the externally recorded potential is nearly identical with the intracellularly recorded potential. Thus, Håkansson (1957*a*) found that when the medium around the fiber was an approximately 2-μ layer of Ringer's solution, the potential recorded from the surface of the fiber had a voltage of about 120 mV, that is, the same as that of the intracellularly recorded potential. The reconstructed monophasic potential has a shape similar to that of the intracellularly recorded potential, but its duration is slightly shorter (2.9 ± 0.20 msec., as compared with 3.6 ± 0.25 msec., measured from 50 per cent rise to 50 per cent fall, at 21° C.). As expected, the conduction velocity is lower by one half than that of the fiber immersed in a large volume of Ringer's solution.

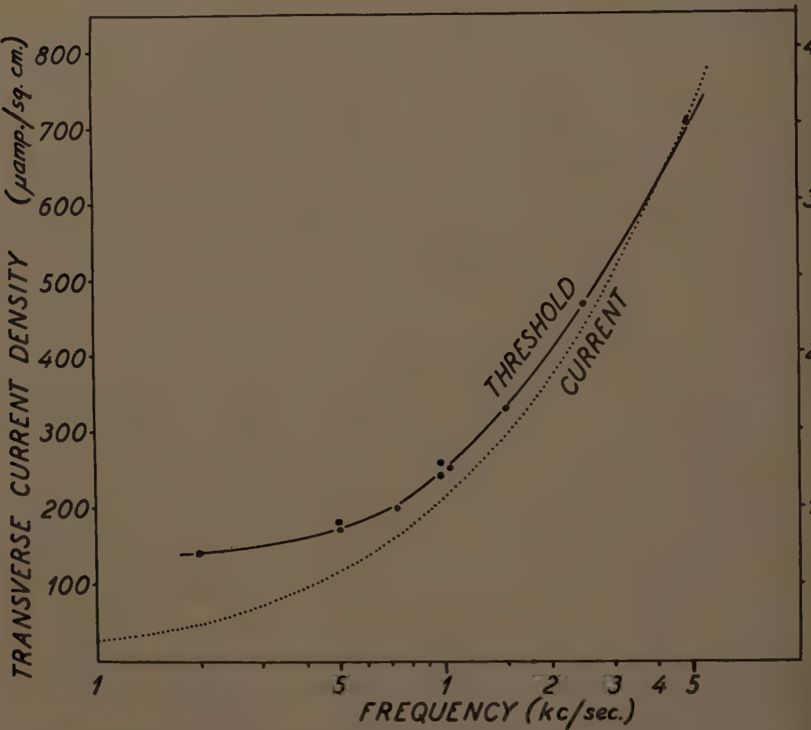

FIGURE 14. Transverse current density (*left ordinate*) and contraction threshold (*right ordinate*) as a function of the frequency of the transverse AC field (logarithmic scales, 20° C.).

When, on the other hand, the surrounding medium has a low resistance, as when the fiber is immersed in a large volume of Ringer's solution, the extracellularly recorded potential is diphasic and has a much shorter duration and a much lower amplitude than the intracellularly recorded potential. It terminates shortly after the peak of the monophasic intracellularly recorded potential. It has not been possible to express the exact relationship between the intracellularly and the extracellularly recorded potentials, but the relationship is described with good approximation by

$$\phi = \frac{r_e A}{4\pi r_i} \int_{-\infty}^{+\infty} \frac{\delta^2 V_i}{\delta z^2} \frac{dz}{\sqrt{r^2 + (z - z_1)^2}}$$

where ϕ is the extracellularly recorded potential, r_e the external longitudinal resistance per centimeter, r_i the internal longitudinal resistance per cm., A the cross-sectional area of the fiber, V_i the intracellularly recorded action potential, r the distance from the fiber axis at which the potential is recorded, and z the distance along the fiber axis (Rosenfalck, 1957).

This expression derives from application of volume-conductor analysis to a cylindrical current source as applied by Lorente de Nó (1947) to nerve. According to this expression, the extracellular potential is roughly proportional to the second derivative of the intracellular potential, that is to its change in slope per unit time. That the extracellular potential in muscle fibers in contrast to the findings on nerve does not display a third positive phase is due to the protracted course of repolarization of the intracellular potential in muscle. This causes the change in slope to be gradual and, therefore, the second derivative to be so small that the third phase is masked by the noise level of recording. Outside a whole muscle in Ringer's solution the third positive phase of many fibers is summed to give the recorded potential its triphasic form, as in nerve (Craib, 1928). In single muscle fibers *in situ* the extracellular potential is triphasic on account of the high external resistance.

The spread of the equipotential surfaces was determined (Håkansson 1957*a*) from the action potential recorded from the single muscle fiber at different distances in Ringer's solution. The equipotential lines for the positive and negative phases of the potential spread almost symmetrically around the zero line. For potentials exceeding 100 μV, the longitudinal spread was 0.7 mm. to each side from the zero potential point and the transversal spread 0.3 mm. from the fiber surface.

The maximum current density of the action currents flowing perpendicularly to the equipotential lines was 0.3 mAmp./sq. cm. and the total current through the membrane $9 \cdot 10^{-6}$ A. With a duration of the propagation of 5 msec. per cm. fiber length, the total electric charge passing through the membrane was $4.5 \cdot 10^{-8}$ coulomb. For a fiber 100 μ in diameter this corresponds to a total charge passing the membrane in one direction of $1.4 \cdot 10^{-6}$ coulomb per sq. cm., which, in turn, corresponds to the transport of $1.4 \cdot 10^{-11}$ mole univalent ions per sq. cm. In single nerve fibers the flux was 3 to 5 times smaller (Keynes and Lewis, 1950).

To clarify the effect of the high external resistance around fibers *in situ*, a comparison was made of the intracellularly recorded potential from a deep fiber *in situ* with the extracellularly recorded potential of the same fiber (FIGURE 15). The stimulus was confined to one fiber by using an intracellular stimulating electrode. The voltage of the extracellularly recorded potential was considerably lower than that of the intracellular potential and decreased, with decreasing external resistance, being 6 to 7 mV just outside deep fibers *in situ* and 1 mV just outside a fiber in Ringer's solution. The fact that the external resistance around fibers *in situ* is of the same order as the internal resistance of the fiber ($3 \cdot 10^6$ Ω/cm./100-μ fiber; Katz, 1948) and, therefore, about 100 times larger than that around the fiber in Ringer's solution, results in a difference in conduction velocity under these two conditions as described on p. 425. The high external resistance around an active fiber surrounded by inactive ones is due to the insulating properties of inactive fibers to currents which arise from potential changes of 100 to 300 μsec. duration (Buchthal *et al.*, 1955).

The voltage of the extracellularly recorded potential falls steeply with increasing distance from the axis of the fiber. With an isolated muscle fiber immersed in Ringer's solution the potential was reduced to one tenth of its maximum value within 0.6 mm. distance from the fiber axis (Håkansson, 1957*a*). The relationship between voltage and distance from the source is approximately linear on a double logarithmic scale (FIGURE 16). For fibers of different diameter the relationship is parallel and the voltage (V) varies approximately inversely proportional with distance (r) as $V = kr^{-1.3}$ where r is the distance of the recording electrode from the fiber axis. At the same distance from the fiber axis, voltage increased with fiber diameter as approximately the square of the diameter. Lorente de Nó's (1947) calculations of the action potential recorded from a whole nerve in a volume conductor give a relationship between voltage and distance that is parallel to that found for the isolated muscle fibers, but displaced to larger distances on account of the larger diameter of the current source.

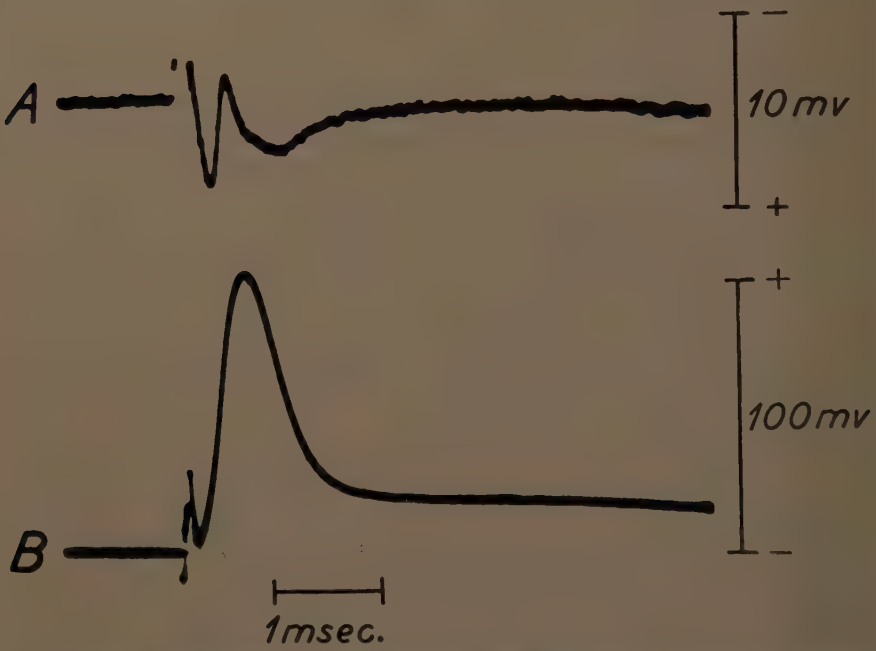

FIGURE 15. Action potentials recorded from a deep fiber of frog's sartorius muscle: *A*, microelectrode placed at the surface of the fiber; *B*, intracellular recording (22° C.).

For action potentials recorded from muscle fibers *in situ*, voltage as a function of distance decreases more steeply than for the fiber immersed in Ringer's solution, one tenth of the maximum being obtained at a distance of 0.45 mm., that is, voltage (V) varied approximately with the inverse square of the distance as $V = kr^{-2.4}$. The much steeper change of voltage with distance for fibers *in situ* is again due to the high external resistance as compared with that around a fiber immersed in Ringer's solution (Buchthal *et al.*, 1957).

Around the cells of the spinal ganglion (Svaetichin, 1951) and of the motor horn (Fatt, 1957) there was a still steeper change of voltage with distance. The action potential disappeared within the noise level at a distance of about 100 μ from the cell which had a diameter of 50 to 60 μ. With a myelinated nerve fiber placed in a thin layer of Ringer's solution, Tasaki and Tasaki (1950) found that voltage within 0.7 mm. from the node had decreased to one third of the maximum value. However, in these measurements the action currents were confined to a single plane, and no direct comparison can be made to measurements in a volume conductor.

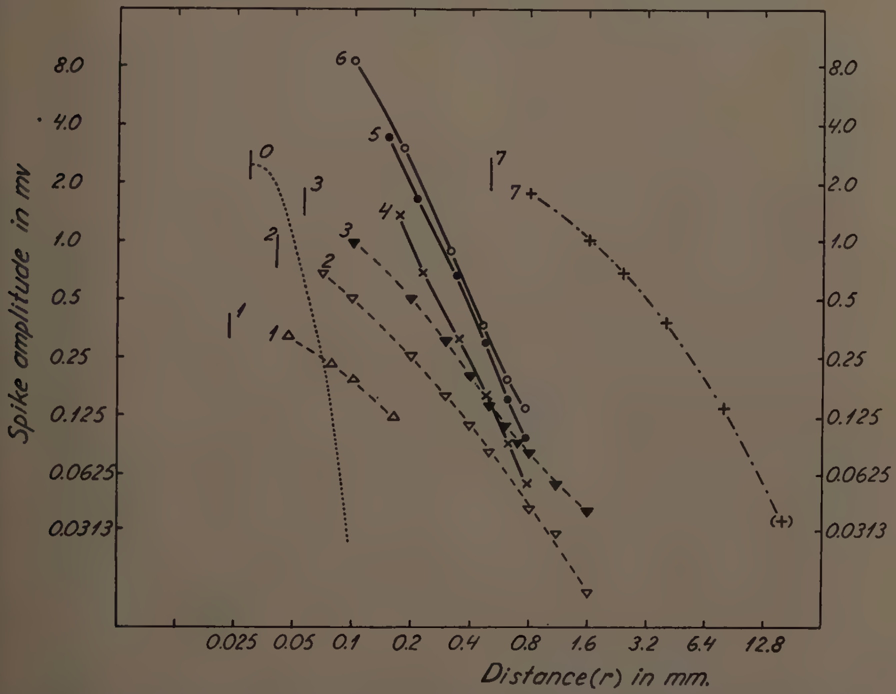

FIGURE 16. Spike amplitude as a function of the distance from the axis of the potential source (logarithmic scales). Key: 1, 2, and 3, isolated frog muscle fiber of 47 μ (1), 82 μ (2), 112 μ (3) diameter (Håkansson, 1957*a*); 4, 5, and 6, spikes of motor unit potentials from human brachial biceps; 7, calculated from Lorente de Nó's theoretical data on nerve (1947); 0, spinal ganglion cell (Svaetichin, 1951). From Buchthal *et al.*, 1957; reproduced by permission from *Acta Physiologica Scandinavica*.

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LOCAL ACTIVATION OF MUSCLE

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The communications of H. E. Huxley and of H. H. Weber, elsewhere in this monograph, deal with muscle contraction from the structural and chemical points of view, respectively, while that of F. Buchthal is concerned with excitation and the propagation of the muscle action potential. My own contribution has to do with a step that lies between the action potential and the contraction process itself, representing a part of the sequence of events (for there must be several such steps) usually grouped under the name of "excitation-contraction coupling." Only a short account of the experimental work will be given here, since it has recently been published in full (Huxley and Taylor, 1958).

The action potential consists in a short-lasting change (either a reduction or a reversal) of the potential difference that exists across the resting cell membrane. If this change took place simultaneously at all points on the surface of the fiber, there would be no potential gradients within the fiber interior at any time; indeed, there would be no electric change that could produce a response in any kind of detector that was located entirely in the interior of the fiber. On the other hand, the potential gradient in the thickness of the membrane itself (and extending for a few angstrom units into the aqueous phases on each side of it) changes by some 10^5 V/cm., an amount sufficient to cause a high degree of orientation of dipolar molecules or to move charged particles from one side of the membrane to the other. This concentration of the electric effects in the substance of the membrane suggests that it is the change of membrane potential itself that initiates the next step in the sequence of events leading to contraction, but it must be remembered that the assumption that the change in potential takes place simultaneously all over the fiber surface is not usually justified. During the normal propagation of an action potential (in frog muscle), the potential gradient within the fiber substance is about 3 V/cm. and lasts for 0.1 to 0.2 msec., and it is necessary to consider the possibility that this field, small as it is (or the current associated with it), is the component of the propagated action potential that is directly involved in initiating contraction. The attractiveness of this possibility lies in the fact that this potential gradient exists throughout the substance of the fiber, so that there would be no need to postulate a further link of the kind that is necessary if a change in the membrane has to influence myofibrils many microns away in the middle of the fiber. Although the experiments that Csapo reports in this monograph suggest that longitudinal currents in some conditions may be able to cause contraction, the evidence is that potential gradients of this order do not do so by themselves (Sten-Knudsen, 1954; Watanabe and Ayabe, 1956). On the other hand, much positive evidence indicates that membrane potential change is the essential factor. Thus, (1) contraction occurs when membrane potential is lowered by any of a variety of different treatments (Kuffler, 1946), such as application of current, solutions with raised potassium concentration, or acetylcholine at the end plate; (2) contraction due to high K concentration can be inhibited locally by anodal current flow (Kuffler, 1946); (3) transverse current causes a muscle fiber to shorten more on the side where membrane potential is reduced than on the side where it is raised. These responses are graded with the degree of the depolarization because the all-or-none electric response of the membrane has been eliminated; if the depolarization is of short duration, the mechanical response may have the time course of a normal twitch (Brown and Sichel, 1936) in spite of the fact that its amplitude is graded. The apparent difference between these "contractures" and the normal twitch or tetanus contractions is therefore attributable to the differences in the membrane potential change, and is no reason for supposing that the steps leading on to contraction are not the same.

If we accept the view that the membrane potential change is the component of the spike that is causally linked to the contraction, we are immediately faced with the question how this change influences myofibrils that may be as much as 50 μ away from the membrane where the large potential gradients exist. The simplest possibility, that a substance liberated at the membrane during the spike diffuses inward to activate the myofibrils, was eliminated by A. V. Hill, who showed (1948) that the time required for diffusion was too great for the speed (Hill, 1949) with which the contractile material of frog muscle is activated. More rapidly spreading physicochemical processes occurring in the bulk phase of the interior of the fiber could be imagined [for example, a reaction catalyzed by its own products, or something resembling crystallization (Hill, 1949)], but it is difficult to think of any such process that would not behave in an all-or-none manner, while the steps between electric change and contraction appear to be graded (the all-or-none character of a twitch being imposed by that of the action potential itself). This suggested that there might be specific structures for conducting an influence inward from the membrane; if so, they probably would be placed at definite positions in relation to the striation pattern. The particular hypothesis that was in the minds of R. E. Taylor and myself when we first tried to stimulate an isolated fiber through a small pipette in contact with its surface was that the structures in question might be the Z membranes, consisting of the dense Z lines in the fibrils, together with the tenuous connections that have often been seen with the light microscope between fibrils at the same level. Our initial experiments were on fibers from the frog and fitted in well with this hypothesis (Huxley and Taylor, 1955*a* and *b*, 1958), but subsequent experiments (Huxley, 1956; Huxley and Straub, 1958) have shown that in two other kinds of muscle, from the crab and the lizard, the inward spread takes place separately in each half sarcomere.

The principle of the experiments was to lower the potential difference across a very small area (1 to 2 μ diameter) of the surface membrane of a muscle fiber, under high-power microscopic observation. This would be expected to cause contraction only if the area depolarized covered the surface attachment of one of the hypothetical structures along which inward conduction might take place. The local depolarization was achieved by placing the end of a glass pipette of the required diameter in contact with the fiber surface and applying a current to the pipette in the direction to make its interior go negative with respect to the surrounding fluid. The currents that enter or leave the fiber through the few square microns of surface that are depolarized in this way are orders of magnitude too small to have appreciable effects on the rest of the fiber; for example, action potentials are not established. Thus, the only effect of the current is to lower the potential at the outside of the membrane within the area covered by the tip; the potentials elsewhere, both inside and outside the fiber, are practically unaffected.

Experiments on isolated twitch fibers from frog muscle were performed both with polarized light and with interference microscopy, the former having the disadvantage that it does not show the Z line well. The results were as follows.

(1) Contraction was never caused when the pipette was opposite an A band.

(2) When the pipette was moved around the fiber in the circumferential direction opposite an I band, positions where a contraction was produced were found at somewhat irregular intervals of about 5 μ.

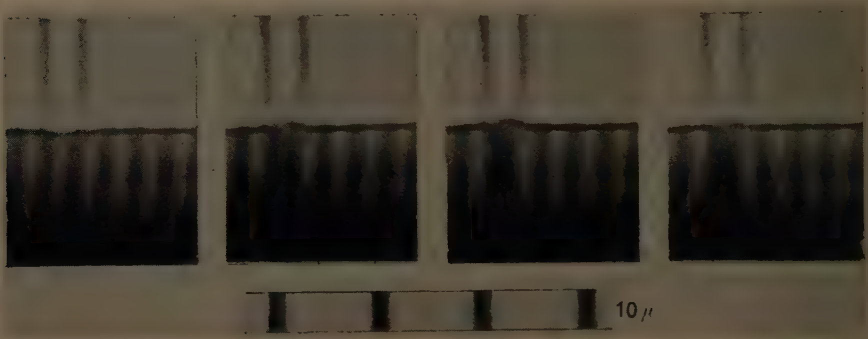

FIGURE 1. Consecutive frames from a motion picture film (16 frames per second), showing the shortening of a single I band in response to a lowering of the potential within the pipette. Isolated fiber from the semitendinosus muscle of a frog. Polarized light, compensated so that the A bands appear dark. Reproduced by permission of *The Journal of Physiology*, from Huxley and Taylor (1958).

(3) When a contraction was produced, it consisted in a shortening of the I band to which the pipette was applied. The adjacent I bands (about 2 μ away) never shortened.

(4) This contraction might spread for as much as 10 μ both inward and in the circumferential direction.

(5) The shortening was always equal in the two halves of the I band; that is, the Z line always remained central.

(6) There was no suggestion of all-or-none behavior.

The general character of the contractions is shown in FIGURE 1.

These results suggest strongly that the inward spread is taking place in two dimensions along some structure in the plane of the Z line.

The next experiments (Huxley, 1956; Huxley and Taylor, 1958) were carried out on crab muscle, in the hope of defining the localization of the sensitive regions more precisely, since the striations in the muscle used were about 8 μ long, as against 3 μ in the frog fibers (which were somewhat stretched). Contrary to our expectation, it was found that no contraction was produced with the pipette opposite either the Z line or the middle of A, but that shortening, confined to the corresponding half of the I band, occurred when depolarization was produced at intermediate positions, that is, near the boundary between the A and I bands. Similar behavior was also seen in lizard muscle (Huxley and Straub, 1958), though the striation pattern was too fine to establish whether depolarization of an extremely small area opposite the Z line was ineffective. These results show that the inward spread is not necessarily associated with the Z line, and also, since both the crab and lizard muscles possess Z lines, that these structures must have some function other than the inward conduction of activation.

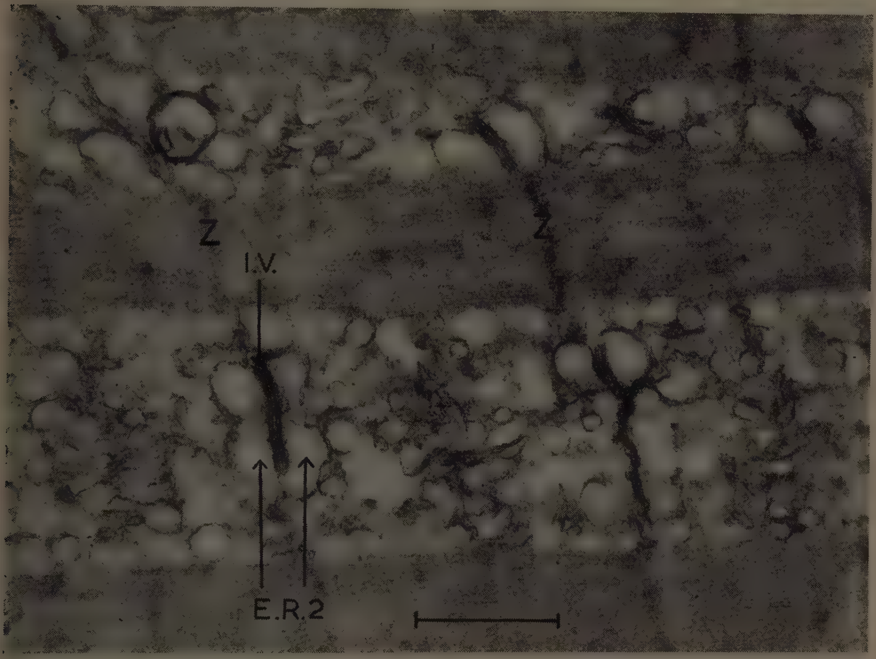

FIGURE 2. Sarcoplasmic reticulum in frog muscle. Triads consisting, in Porter and Palade's (1957) nomenclature, of I-band vesicles (E.R.2) and intermediary vesicles (I.V.) at the level of each Z line. The middle element of each triad appears to be a continuous structure rather than a row of small vesicles. Fixed in buffered 1 per cent OsO_4 ; no further staining. Scale bar, 0.5 μ.

As might be expected, electron microscopy has provided an important suggestion about the structures concerned in this inward spread. In 1953, Bennett and Porter described a sarcoplasmic reticulum lying in the spaces between the myofibrils, and Bennett (1955) suggested that its function might be to regulate the activity of the fibrils. More recent studies, notably by Porter and Palade (1957), have shown much detail in this material. Both in amphibian and in mammalian muscle, it contains elements referred to as triads, but these are disposed differently in the two cases. In muscle from *Amblystoma* (the amphibian used by Porter and Palade) and in the frog (FIGURE 2), the triads occur at the level of the Z line (that is, at the level where we have shown that inward conduction occurs in the frog); in mammals, however, Porter and Palade found 2 triads per sarcomere, placed about one quarter of the way from each Z line toward either of its neighbors. Structures resembling the middle element of a triad had been seen in lizard muscle, with the last-mentioned arrangement, by Robertson (1956), and he too suggested that they were concerned with the inward spread of activation. It was this observation that led Straub and myself to try the local activation experiment on fibers from lizard muscle, and the finding that the two halves of each I band could be made to contract separately by placing the pipette appropriately shows that here, as well as in frog muscle, the part that responds as a unit is centered on a triad (we confirmed with the electron microscope that there are 2 triads per sarcomere in lizard muscle (see FIGURE 3) just as was found by Porter and Palade in mammals). This agreement between the localization found in the local activation experiments and the distribution of the triads as seen in the electron microscope suggests very strongly that activation is conducted inward by some

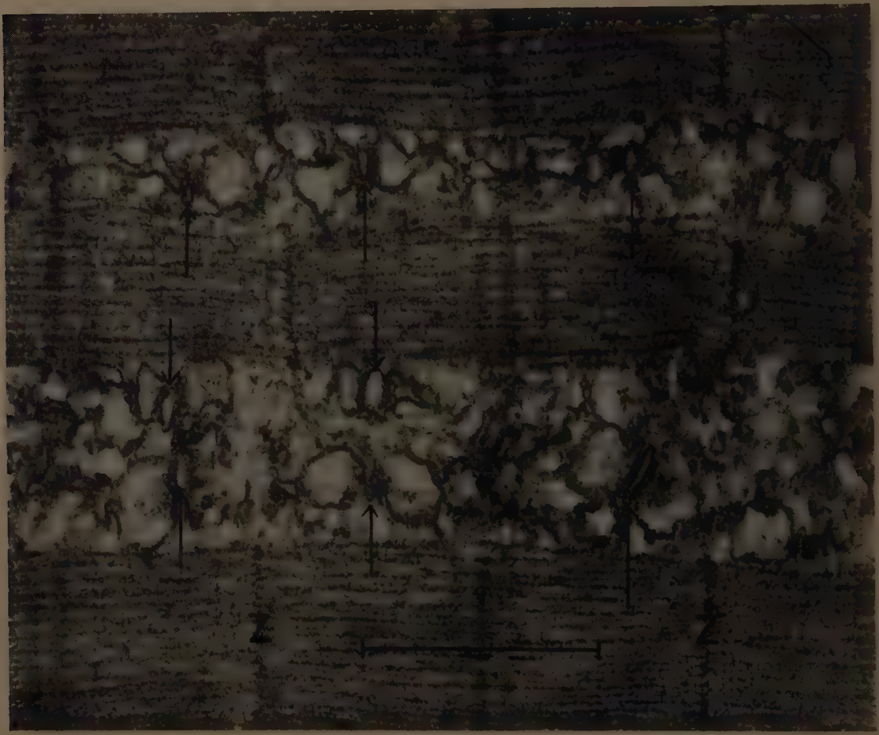

FIGURE 3. Sarcoplasmic reticulum in lizard muscle. The middle elements of several triads are indicated by arrows. They are placed about a quarter of the way from the level of each Z line toward its neighbors, and they are seen to be flanked by larger vesicular elements forming triads as described by Porter and Palade in muscles of other vertebrates. Fixed in buffered 1 per cent OsO_4; stained with phosphotungstic acid. Scale bar, 0.5 μ.

component of the triad, but which component it is cannot be decided on present evidence. For example, it is not clear which, if any, of the components of the triad are in continuity across the fiber; thus, according to Porter and Palade's description of *Amblystoma* muscle, continuity exists not in the triads but in the reticulum at the level of the middle of the A bands; furthermore, the middle component of each triad consists of a row of separate small vesicles. On the other hand, some (see FIGURE 2) but not all of our micrographs of frog muscle show the middle component as a continuous structure as far as can be seen in a single section. Both of these aspects of the question of continuity require further investigation.

As to the mechanism of the inward spread, the only suggestion that has so far been made is that it is electric in nature, assuming continuity in some tubular component. Bennett (1955), Porter (1956), and Edwards *et al.* (1956) all proposed that something analogous to impulse conduction took place along components of the sarcoplasmic reticulum. Our results show, however, that the inward conduction differs from ordinary nerve or muscle conduction in that it does not take place in an all-or-none manner; therefore, if the conduction is electric, then it is more likely to be a graded passive spread along a cable structure. This has been our working hypothesis in Cambridge (see Huxley, 1957), but it must be emphasized that there is no direct evidence for it, and that the simplest form of the hypothesis, which would make the lumen of the tubules continuous with the extracellular fluid and therefore able to conduct changes of membrane potential inward, is not supported by the present electron microscopic evidence.

Even less can be said about the final stage of spread, from the triads to the contractile material itself. The chief relevant evidence is that this is limited in the longitudinal direction to 1 (lizard and crab) or 2 (frog) half sarcomeres. Possible explanations are that a substance is liberated and destroyed within a short distance, or otherwise prevented from diffusing, or that some change is initiated in structures such as the secondary filaments, whose extent corresponds to the distance of spread, but there is no basis at present for deciding between these, and no doubt many other, possibilities.

Whatever the solutions of these problems may be, the phenomenon of local activation remains of great interest in two ways. From the point of view of muscle physiology, it is one of the missing links between membrane potential change and mechanical response, and this is the aspect that has been discussed in this article. In a wider context, however, it may be regarded as a particular case of the regulation of the activity of structures inside a cell, and it is possible that these studies on muscle will provide valuable suggestions about analogous processes in other types of cell whose activities are less easy to control and to observe.

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STUDIES ON EXCITATION-CONTRACTION COUPLING*

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This report is a summary of the observations of H. Mashima, T. Sakai, T. Suzuki, Dorothy M. Conway, and B. A. Curtis. The experiments were performed on the rabbit uterus and on frog and turtle cross-striated muscle, under conditions and with techniques of considerable variety. The results, which are in good agreement with one another, show that:

(1) The applied longitudinal field effectively activates K-depolarized, nonpropagating muscle without the necessity of a change in membrane potential during the transformation of the muscle from rest to activity.

(2) Muscle can be "uncoupled," that is, rendered inexcitable temporarily and reversibly at a membrane potential close to its normal value by the displacement of a fraction of its labile Ca. This suggests that depolarization and myoplasmic activity are not linked directly, but are connected by at least one intermediate step in which Ca is involved.

(3) The problem of excitation-contraction coupling was clearly stated by Hill (1950), and the subject was reviewed by Sandow (1952) and by Gelfan (1958). Points of view were expressed by Katz (1950) and Huxley (1956), whereas Sten-Knudsen (1954) presented some experimental data and arguments, discussed by us in view of additional information (Csapo and Suzuki, 1958).

The current position, not substantiated by our observations, is that the applied longitudinal field activates only those portions of the nonpropagating muscle that become depolarized during the application of the stimulus. Thus it is believed that activation is mediated by the depolarizing effect of the applied field upon the surface membrane and that currents flowing through the fibers are ineffective. This conclusion is not derived from direct evidence, however, since no one has as yet demonstrated activation in intact muscle by depolarization alone in the complete absence of current flow. As will be pointed out later, the observation that the nonpropagating muscle, subjected to a certain set of experimental conditions, shortens preferentially at portions where the current density is small, whereas depolarization is excessive (as contrasted to portions where depolarization is nil, whereas the current density is large) is by no means evidence for the ineffectiveness of internal currents. Using the same procedures by which the effectiveness of currents in the activation process was thought to be ruled out, one can easily show that the K-depolarized resting muscle is activated by currents at portions where the depolarizing effect of the applied field is definitely excluded by direct measurements with internal microelectrodes.

Our experiments provide us with no conclusive evidence as yet that internal currents effectively participate in the activation of the normal propagating muscle. They provide us, however, with observations which keep this possi-

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bility open. In addition to pressure (D. E. C. Brown, personal communication) and Ca (Niedergerke, 1956), internal currents (Csapo and Suzuki, 1958) and iodide (Curtis and Csapo, 1958) can now be listed as triggering agents, capable of activating muscle, without necessitating a change in membrane potential. These considerations suggest that depolarization is only a first or "priming" step in the series of events leading to myoplasmic activity and that additional steps must be looked for in an effort to understand the coupling process.

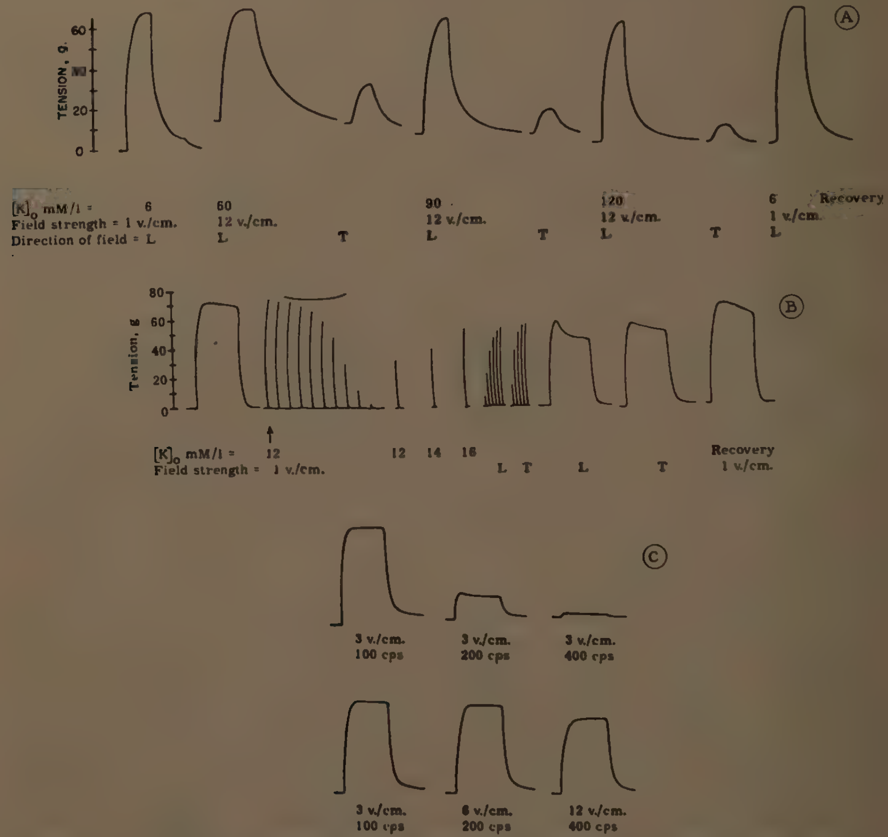

FIGURE 1. (*A*) Rabbit uterus (twenty-seventh day of pregnancy), 37° C. Stimulus: AC 60 cps. The NaCl in the Krebs solution is substituted with KCl, as indicated. Note that the effect of the transverse field is dependent, whereas that of the longitudinal one is independent of the extent of K-depolarization (that is, of the residual membrane potential). Note the normal shape of the mechanical response and complete recovery on repolarization. L = longitudinal field, T = transverse field. (*B*) Frog sartorius, 20° C. Stimulus: AC, 60 cps, 1 to 16 V/cm. Note that after the cessation of the "propagated response" (1 V/cm.), abolished by excess K, tension (elicited by 16 V/cm., L) increases with gradual depolarization. Note that tension (at a $[K]_0 = 16$ mM/l.) is a function of the field strength (2 to 20 V/cm.) in both the L and T fields. Note the normal shape of the mechanical response, and good recovery on repolarization. (*C*) Frog toe muscle, 20° C. $[K]_0 = 32$ mM/l., $[Ca]_0 = 9$mM/l. Stimulus: longitudinal AC. Note the diminishing effect of increasing cycle frequency on tension and that, if the ratio (V/cm.)/cps is kept constant, tension remains constant.

Rabbit uterine strips (FIGURE 1*a*), completely depolarized by excess K of

120 mM/l. (substituting K for Na), develop maximum tension in a longitudinal AC field, but only about 15 per cent tension in the transverse field (Csapo, 1954). Longitudinal DC is also more effective than the transverse. The transverse field effect is dependent on the residual membrane potential (left behind by excess K treatment), whereas that of the longitudinal is independent of it. Thus the "K-primed," completely depolarized uterus can be stored in the resting condition to be maximally activated later by longitudinal, but not by transverse current.

It may be argued that the K-depolarized short fibers of the uterus acquire a membrane potential during AC stimulation, and the results obtained are due to membrane potential changes. However, this is unlikely because the transverse field is ineffective. The use of 10 to 12 V/cm. electric field might be objected to as being unphysiological, but the uterus repeatedly treated by such strong currents develops a mechanical response of normal shape and completely recovers on repolarization in normal Krebs solution.

Frog muscles (FIGURE 1*b* and *c*) develop considerable tension if the $[K]_0$ is increased sufficiently and if both the field strength and frequency of the stimulating AC field are optimal with regard to the orientation of mounting of the muscle. Tension thus produced, which may exceed 50 per cent of the maximal, is difficult to explain by end effects. As long as the muscle is not overstimulated, the shape of the tension curve is normal and recovery on repolarization is good, provided the muscle has been dissected with the utmost care. The best preparation in our experience is the retractor penis muscle of the turtle, not only because it is long and has a relatively uniform diameter, but because it need not be dissected or teased, but only freed at its bony and connective tissue attachments. After having studied in frog muscle the effects of various parameters on tension such as the type of block, the $[K]_0$, the $[Ca]_0$, the field strength and frequency, and the direction of the applied field, we turned to the more suitable turtle muscle and studied its shortening along its length.

Recording of tension alone does not describe activation at different portions along the length of the nonpropagating muscle, nor does it reveal whether the activating effect of the applied field is due to membrane potential change or, rather, to the currents flowing through the fibers. In order to distinguish between the respective effects of these two potential factors, one or the other must be ruled out.

At the cathode, as in the normal propagating muscle, depolarization and current flow coincide. In the middle portion and at the anodal half of the muscle, however, one can expect to observe the effect of current without a disturbing interference of depolarization. Using the intracellular microelectrode technique (FIGURE 2*a*), we now demonstrated that this expectation is justified (FIGURE 2*b*). In the longitudinal DC field the cathodal half of the K-depolarized muscle becomes further depolarized, whereas the anodal half is polarized during the application of the stimulus. The membrane potential in the middle portion is unchanged.

The observation, therefore, that K-depolarized nonpropagating muscle shortens in the longitudinal DC field at its middle and anodal portions is evidence for the triggering effect of internal currents (FIGURE 3*b*). These measurements with microelectrodes rule out the possibility that the effect of the longi-

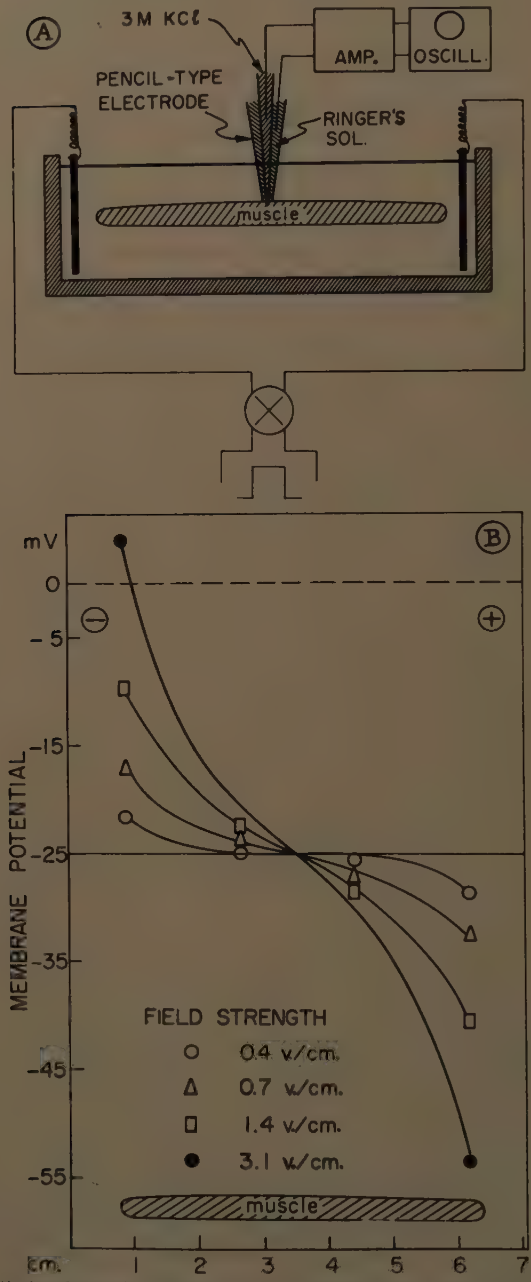

FIGURE 2. (*A*) A schematic illustration of the method used for measuring the changes in membrane potential along the length of the K-depolarized (nonpropagating) muscle during the application of a longitudinal DC field. Note the double electrode. (*B*) Turtle retractor penis. 20° C., $[K]_0 = 24$ mM/l. Stimulus longitudinal DC, of different strength. Note that the cathodal half is always depolarized, while the anodal half is always polarized during the application of the field, leaving the membrane potential unchanged in the middle portion. Also that the extent of the membrane potential change is a function of field strength.

tudinal field is due to distortion of currents by irregularities in the individual fibers or in the space between fibers. Such a distortion of current would be manifested by depolarization at the middle and anodal portions, an effect that we have not observed. Furthermore, if the effect of the longitudinal field was mediated by depolarization (due to distortion of the applied current), the transverse field should be considerably more effective than the longitudinal. In fact, just the opposite is true, as shown by experiments on turtle muscle,

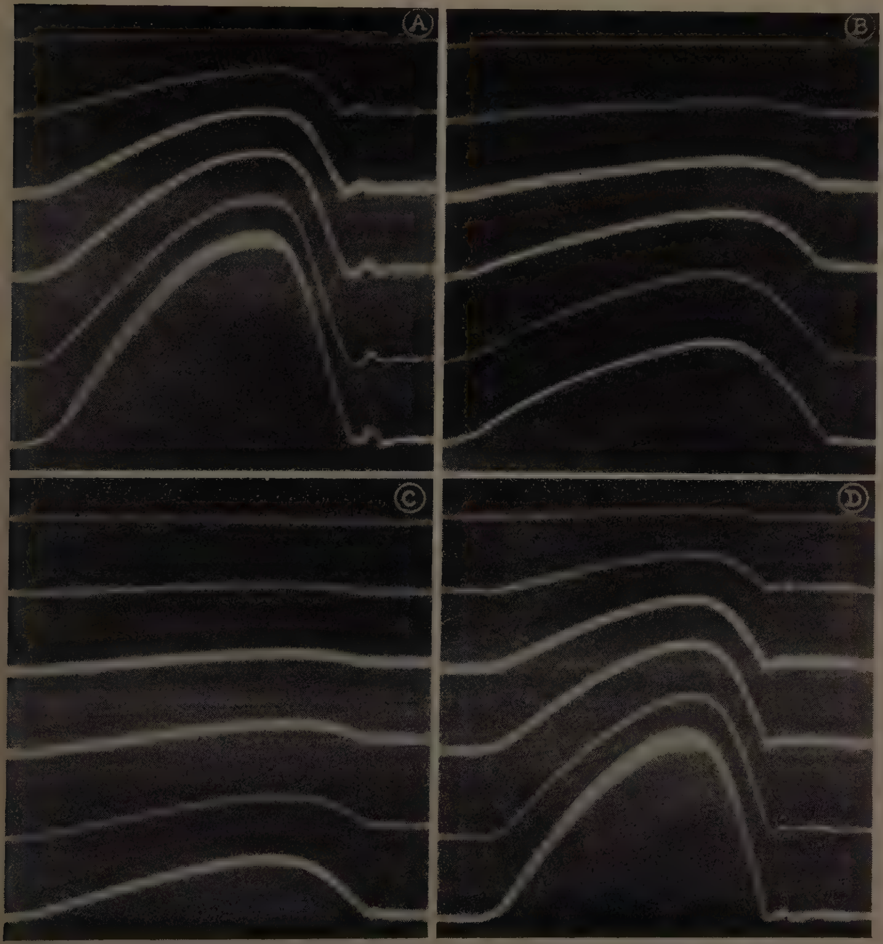

FIGURE 3. Turtle retractor penis muscle. 20° C., afterloaded with 3 g. The muscle is marked off with a fluorescent dye and the movement of the marks, during shortening, is recorded by a constant-speed motion picture camera. (*A*) Propagating muscle in normal Ringer's solution. Stimulus: 1 V/cm., AC, 60 cps. Note that all portions shorten to the same extent. (*B*) Nonpropagating muscle, $[K]_0 = 20$ mM/l. Stimulus: longitudinal DC, 6 V/cm. Note shortening in the middle and anodal portions, and also the normal shape of the mechanical response. (*C*) The same as *B*, but the field is transverse. Note the slight effect. (*D*) Propagating muscle, after recovery in normal Ringer's solution. Note the good recovery.

which can be more extensively depolarized by excess K than the frog muscle (FIGURE 3*c*).

We have developed (Mashima and Csapo, 1957) an accurate and simple method for measuring shortening in the nonpropagating (afterloaded) muscle along its entire length. The muscle is marked off with a nontoxic fluorescent dye (RCA material: No. 33-Z-607), and the movement of the illuminated marks is recorded by a constant-speed motion picture camera (FIGURE 3).

Using this technique with the turtle muscle, which remains functional in spite of excessive and sustained depolarization, it is easy to show that the K-depolarized, nonpropagating ($[K]_0$ = 16 to 24 mM/l.) muscle shortens along its entire length (although little if at all at the extreme anodal end) when stimulated in a longitudinal DC field. The effective field strength under these conditions is 4 to 8 V/cm. Since shortening at the middle and anodal portions occurs without depolarization (the membrane in fact becoming slightly polarized at the anodal half), it must be the current flowing through the fibers that triggers activity. The mechanical response of these preparations is of normal shape, and recovery on repolarization is complete. These facts rule out an unphysiological effect of the stimulating current, although the field that is applied is stronger than that generated by the action potential. A possible explanation of the concept that sustained depolarization, with excess K, renders the muscle less sensitive to stimulating current than sudden and short-lived depolarization, is presented below.

When the longitudinal DC is abruptly applied, the muscle shortens along its entire length, but cathodal shortening dominates, specifically at field strength 2 to 4 V/cm. If the current is gradually increased, however, the cathodal end hardly shortens, while shortening at other portions is unchanged. Thus the rate of membrane potential change is critical for the effect at the cathode, whereas shortening at the middle portion of the muscle is dependent only on the applied current and independent of the membrane potential change (FIGURE 4*a*).

These experiments are easy to perform. It is considerably more difficult to obtain shortening curves of good symmetry and, on reversing the polarity of the stimulating current, a mirror image of the shortening curve. The regularity of shortening along the length of the muscle seems to depend on cross-sectional uniformity, a property that muscles seldom possess. The result of cross-sectional nonuniformity, in a partially activated muscle, is asymmetrical shortening along its length. The thicker portions exert tension on the thinner ones. One of us (H.M.) learned to overcome this difficulty by ligating and cutting 6 to 8-cm. portions (of uniform thickness) of about 15-cm.-long turtle muscles. Shortening in these preparations was more symmetrical than in intact muscles, and recovery after short experiments was good. That the cut ends function well is illustrated by the experiment in which the muscle was blocked by Na lack, combined with excess K treatment (FIGURE 4*b*). The muscle shortens along its entire length in the longitudinal DC field and, when the polarity is reversed, a mirror image of the same curve is obtained (FIGURE 4*b*). The longitudinal AC effect is the sum total of that of 2 DC stimuli of opposite signs, as shown by experiments in both the frog toe and turtle retractor muscles (FIGURE 4*c* and *d*).

A rather significant point to consider is the well-known observation that, at field strength $\cong$ 2–4 V/cm., the cathodal effect dominates the shortening of the K-depolarized muscle. Since at the cathode depolarization is excessive, while the current density is small, this observation has been interpreted (Sten-Knudsen, 1954) to mean that depolarization is the responsible factor for myoplasmic activity, whereas the currents flowing through the fibers are ineffective.

That depolarization is an essential first step in the series of events leading to

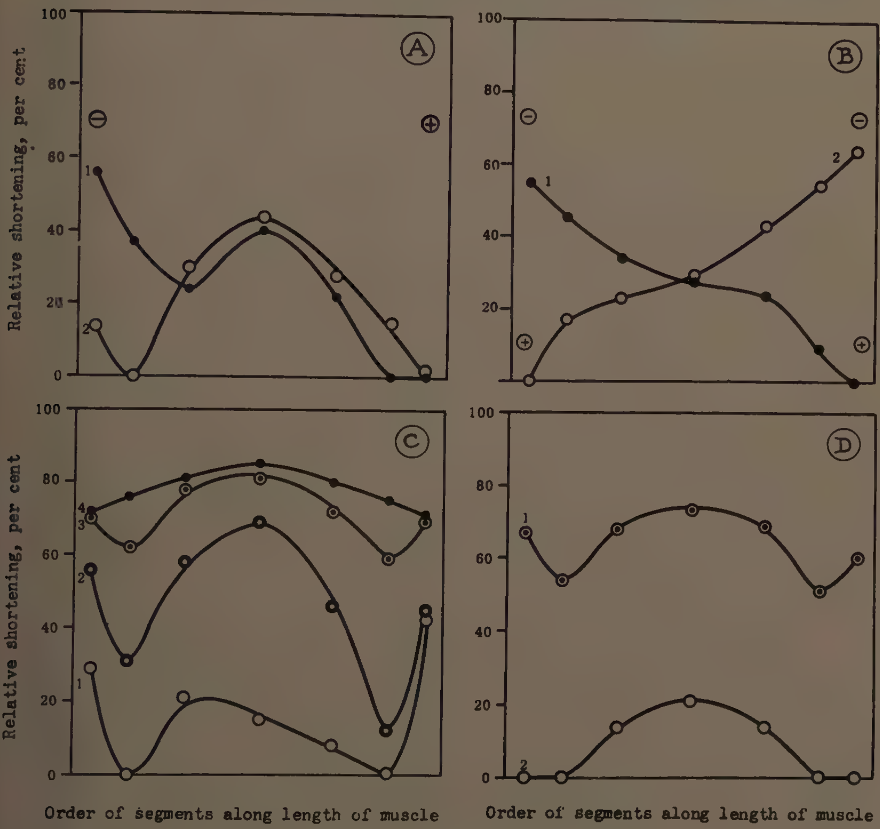

FIGURE 4. *A*, *B*, and *D*, turtle muscle; *C*, frog toe muscle. 20° C.

$$\text{Relative shortening} = \frac{\text{shortening of the nonpropagating muscle}}{\text{shortening of the propagating muscle}} \times 100$$

(*A*) $[\mathrm{K}]_0$ = 20 mM/l. Stimulus: 6 V/cm., DC, longitudinal. The current strength is increased: 1, abruptly; 2, gradually. Note that the cathodal end is dependent, while the middle portion is independent of the rate of the membrane potential change. (*B*) 120 mM/l. NaCl is replaced by choline chloride. $[\mathrm{K}]_0$ = 20 mM/l. Stimulus: 10 V/cm., DC, longitudinal. Change in polarity: 1 and 2. Note that, in spite of the ligated and cut ends, the ends of the muscle function well and the shortening curve changes accurately with a change in polarity. (*C*) Frog toe muscle, $[\mathrm{K}]_0$ = 16 mM/l. Stimulus: longitudinal AC; 1, 2, 3, and 4 = 3, 6, 12, and 16 V/cm. Note that the stronger the field, the greater is the shortening at the middle portion. (*D*) $[\mathrm{K}]_0$ = 16 mM/l. (1), and 24 mM/l. (2). Stimulus: 4 V/cm., AC, 60 cps, longitudinal. Note that the higher the $[\mathrm{K}]_0$, the less is the end effect.

activity (Kuffler, 1946) can hardly be doubted. In all our experiments we could trigger effectively nonpropagating resting muscles by longitudinal current only if prior to the application of the field we depolarized the muscle sufficiently with excess K. Blocking agents, such as procaine or Na-lack, which abolish conduction at a high membrane potential, provide a preparation that can be stimulated only very ineffectively even with strong currents, whether longitudinal or transverse. The sensitivity of the muscle to current can be greatly improved by moderate depolarization with excess K.

On the other hand, we have shown that the triggering effect of current need not coincide with depolarization during the transformation of a resting, nonpropagating muscle to an active one. A series of experiments on the contracture of the turtle muscle (Conway and Csapo, 1958; Curtis and Csapo, 1958) also led us to the conclusion that this transformation can be achieved without change in membrane potential. In fact, slight polarization during activation is permissible, provided the muscle has been primed by excess K treatment.

The turtle muscle develops 2 kg./sq. cm. tension or more in contracture elicited by excess K, a value comparable to that of isolated single fibers (Horowitz and Hodgkin, 1956). This high value suggests that diffusion does not hinder contracture to any great extent in this preparation. It was of interest for us to find that, when contracture reached a maximum value upon immersion of the muscle in excess K, we could superimpose tetani upon the contracture by rather weak (1 or 2 V/cm.) electric current. The response to electric stimulation declined at a rapid rate, but could be prolonged by excess Ca. We thought that this observation suggested that depolarization triggers a subsequent step that favors activity by rendering the muscle sensitive to current, a reaction that has a rapid rate of dissipation and is Ca-sensitive. As yet we have no evidence that this explanation is the only and the correct one, but if it is, the cathodal response, as compared to that of the middle portion in the K-depolarized muscle, can be well understood.

By placing the muscle in excess K of 14 to 24 mM/l., we render it nonpropagating and sensitize it to the triggering effect of current. However, because depolarization is only partial and is sustained, the sensitivity of such a resting muscle to current is poor, not at all comparable to an intact propagating muscle that becomes completely depolarized for only a very short period of time. If we apply a weak longitudinal current to the K-depolarized muscle, the cathodal end becomes completely depolarized for a period of 0.5 to 1 sec. (during stimulation), and thus may attain a state similar to that of a propagating muscle when stimulated. On the other hand, depolarization does not occur at the middle portion, and the anodal half becomes somewhat polarized. The sensitivity of the muscle to current, as indicated by the extent of activation, is quite different, therefore, at the cathode, in the middle, and at the anodal half. The point, which may turn out to be very significant, is that it is not depolarization per se that is the decisive factor in potential activation, but rather the consequences of depolarization, particularly a step subsequent to and quantitatively dependent on its extent. If the step subsequent to depolarization sensitizes the muscle to current, and thereby links membrane and myoplasmic events, it may be quite meaningless to calculate the field strength and current generated by the action potential in the normal propagating muscle and compare these values with those applied to the nonpropagating muscle in triggering it, because the sensitivity of the two preparations to current may be drastically different.

The following observations made in the course of our K-contracture studies might be of interest. If the muscle is rendered nonpropagating before treatment with excess K (by K, procaine, or Na-lack), the contracture tension is reduced (FIGURE 5*a*). If the excess K is applied gradually rather than suddenly, no contracture develops at any external [K], which suggests that a true "threshold" does not exist. The lack of contracture in case of a gradual depolarization may be explained by "accommodation" and a subsequent loss of contracture potency. Contracture potency is not lost, however. When the muscle is treated by excess K 10 to 20 mM/l. higher than the so-called "threshold" value (25 mM/l.), a sudden increase of excess K does elicit contracture (FIGURE 5*b*). It appears that the magnitude and the rate of change of membrane potential are as critical as the value to which it is reduced.

Once the muscle is primed by excess K it can be triggered by NaI without a change, or even in spite of a small increase in membrane potential (FIGURE 5*c*). Contracture can be repeatedly obtained in the K-primed turtle muscle without repolarizing its membrane, by introducing, withdrawing, and reintroducing iodide into the Ringer's solution (FIGURE 5*d*). Thus iodide not only prolongs, but, in the K-primed muscle, initiates the active state.

After short treatment with moderate K excess, the K-contracture is inversely proportional to temperature (FIGURE 5*f*) and the $[Ca]_0$ (FIGURE 5*e*).

For the sake of exposition it may be helpful at this point to present a working hypothesis that helped us in designing the experiments that follow. Myoplasmic activity may arise from the joint effects of depolarization and current flow. Depolarization can be looked upon as a priming step, altering the muscle in such a way that a triggering agent could be effectively moved by current flowing through the fibers. The "threshold" current is determined by the rate, extent, and duration of depolarization. Depolarization liberates a muscle substance; the greater the depolarization, the more triggering substance is released. If depolarization is slow and sustained, however, the triggering substance escapes from the vicinity of active sites more in one muscle (frog) than in others (rabbit uterus). This escape renders the tissue insensitive to electric stimulation.

The crucial question, of course, is the nature of the "activating" muscle substance. We should like to propose (not as an original suggestion—see Sandow, 1952) that this critical substance is the Ca ion or a muscle substance activated by Ca. This possibility has been implied already by some of the observations presented thus far. We now present some further evidence in support of it.

Immersion of the frog sartorius in a 24 mM/l. excess-K, Ca-free Ringer's solution quickly renders the muscle completely unresponsive to electric stimulation (FIGURE 6*a*). The response to strong currents, however, returns if the $[Ca]_0$ is increased and tension becomes the function of the Ca excess. This effect of Ca cannot be accounted for by the known effect of Ca on the membrane potential (Jenerick and Gerard, 1953) and it can be shown (FIGURE 6*b*) that tension in the nonpropagating muscle at the same membrane potential (for

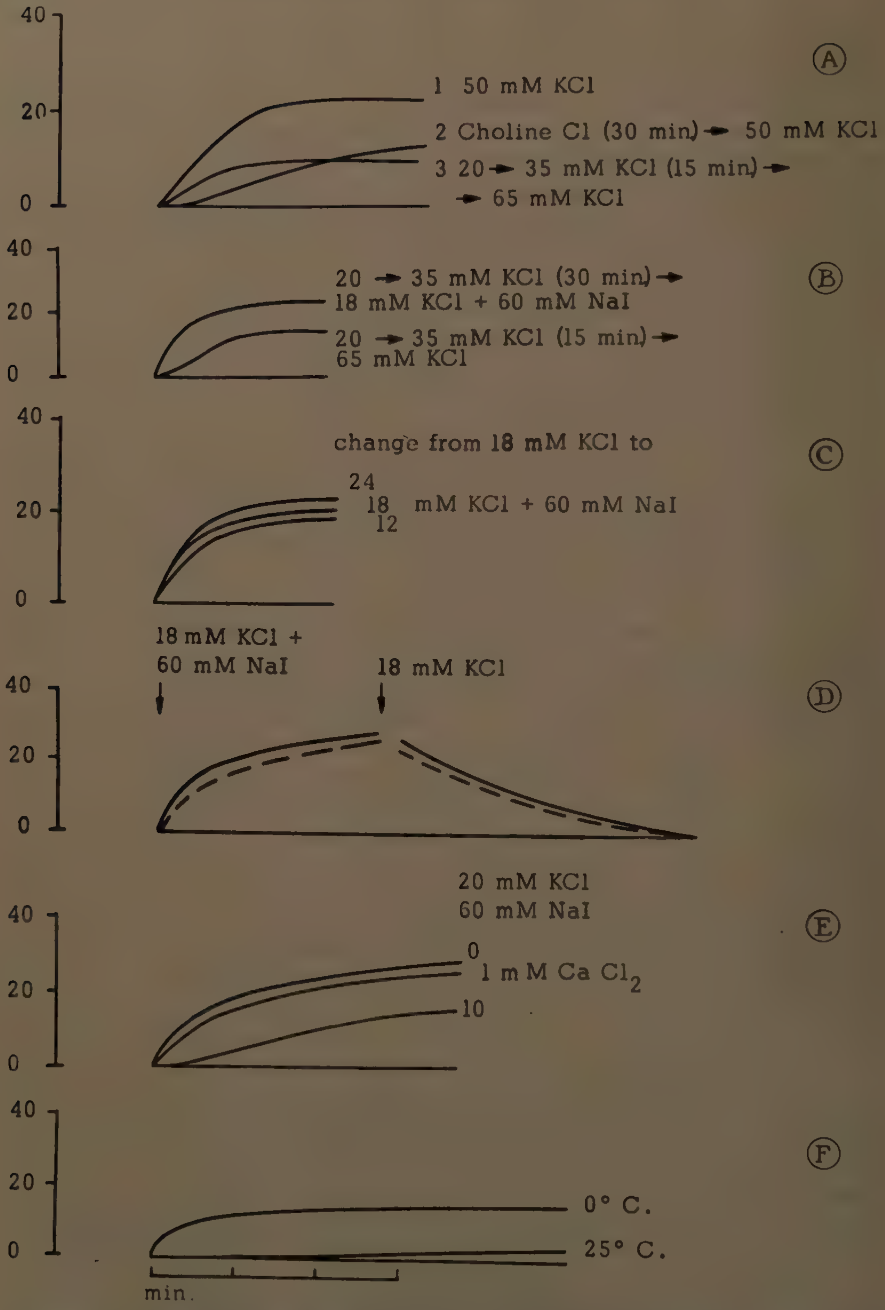

FIGURE 5. Turtle, retractor penis, 20° C. (*A*) Pretreatment with choline chloride (30 min.) or with 35 mM/l. KCl (15 min.) decreases contracture tension, elicited by sudden immersion of the muscle in excess [K]. (*B*) Muscle pretreated with KCl (35 mM/l.) develops more contracture tension if treated with NaI, in spite of an increase in its membrane potential

example, 40 mV) is a function of the external [Ca]. It can also be demonstrated that at a low membrane potential, for example 35 mV, activation is greater if this value is reached from above rather than from below.

These observations can be explained by the assumption that Ca is rather strongly bound in the intact muscle, but is released as a function of the extent of depolarization. The appearance of ionized Ca favors activity. If depolarization is sustained, however, Ca escapes from active sites because it moves out with the gradient. If the Ca gradient is decreased by increasing the $[Ca]_0$, the outward movement of Ca can be controlled (within limits) and activity maintained. The escape of Ca is a relatively rapid process as compared with its readsorption, and thus recovery from complete inexcitability is very slow.

Turtle muscle washed repeatedly with Ca-free Ringer's solution of normal [K] = 2.5 mM/l. maintains normal excitability for hours. However, if this treatment is combined with depolarization with excess K, excitability is lost and recovery in normal Ringer's solution is greatly delayed. The delay in recovery is a function of (1) the extent of depolarization (FIGURE 7*a*), (2) the $[Ca]_0$ during depolarization (FIGURE 7*b*), and (3) the $[Ca]_0$ during recovery (FIGURE 7*c*).

Ca measurements indicate no substantial Ca loss after the treatment of the muscle with Ca-free Ringer's solution containing excess K. This suggests either that Ca may be displaced only from "active sites" or that the structure (sarcoplasm?) in which true Ca loss occurs has a small volume in relation to that of the muscle.

Recovery in normal Ringer's solution after K-depolarization is complete, but slow. Recovery is incomplete in Ca-free Ringer's solution, and its rate is increased by excess $[Ca]_0$. It is significant that the membrane potential of the recovering muscle in normal Ringer's solution does return considerably more quickly than the propagated muscle response (FIGURE 8*a* and *b*). During a period of several minutes (determined by the conditions of the experiment) the muscle is "uncoupled"; that is, its membrane potential is above the "critical" value, yet its propagated response fails. This period is shortened by excess $[Ca]_0$.

We have observed (Csapo and Wilkie, 1956) that the frog sartorius, treated with excess K of only 24 mM/l., recovers in normal Ringer's solution very slowly at low temperature (3° C.). Furthermore, a sudden increase in temperature of the recovering muscle brings about prompt recovery. The membrane potential at low temperature recovers more quickly than does propagation (Wilkie, personal communication).

We have repeated and extended these experiments, using the turtle muscle. We found that the membrane potential of the muscle recovering at low tem-

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(KCl = 18 mM/l!), than if further depolarized with KCl = 65 mM/l. (*C*) Muscle pretreated with 18 mM/l. KCl develops contracture when treated with NaI. The tension developed is little altered by a change in membrane potential of opposite directions, elicited by increasing or decreasing the external [K]. (*D*) By the addition and withdrawal of NaI, contracture is initiated and abolished, respectively, in the excess K treated muscle (18 mM/l.), without repolarization and recovery. (*E*) Contracture tension elicited by NaI, in a muscle pretreated for 3 min. with a Ringer's solution containing excess [K] = 20 mM/l. and of different [Ca], is inversely proportional to the $[Ca]_0$. (*F*) Contracture tension, elicited by NaI (60 mM/l.) and excess KCl (14 mM/l.), is inversely proportional to the temperature.

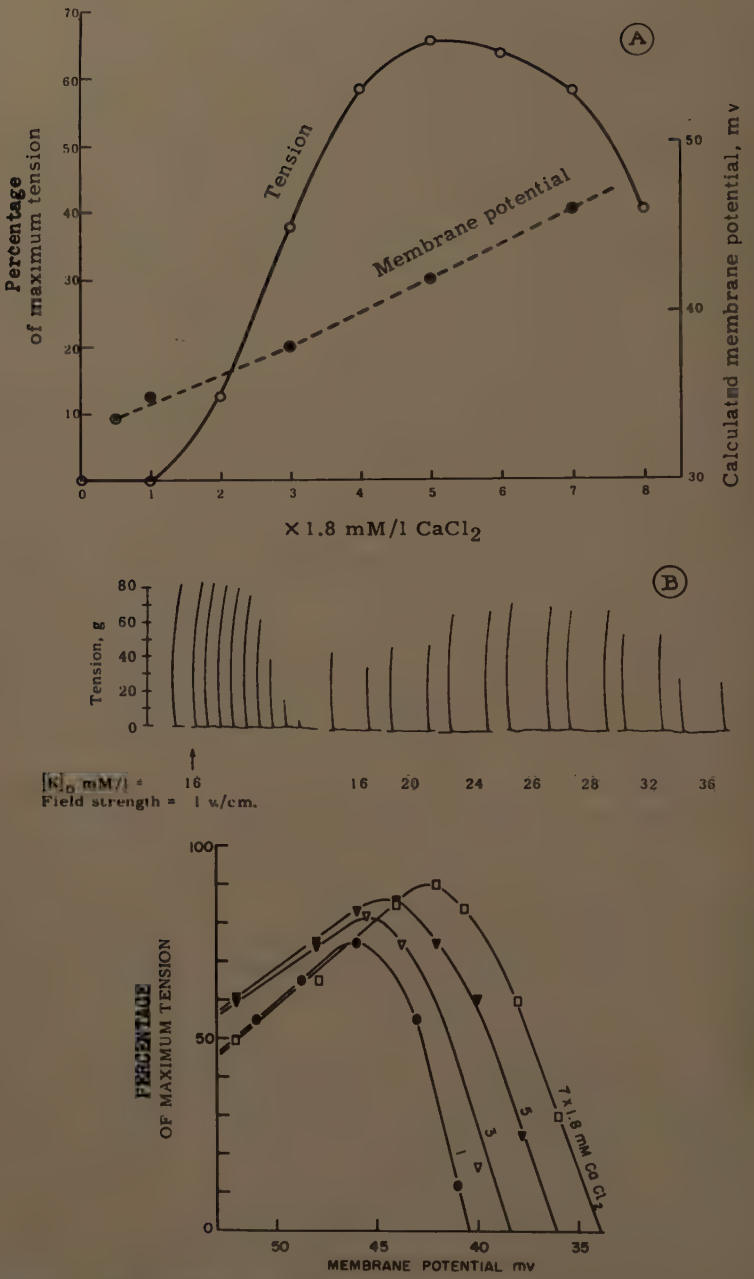

FIGURE 6. Frog sartorius, 20° C. (*A*) The muscle is depolarized by excess [K] of 24 mM/l. in Ca-free Ringer's solution for 30 min. The muscle quickly ceases to respond to electric stimulation, even if the strength of the longitudinal AC field is 16 V/cm. Note that excess Ca restores the mechanical response and that, when tension had increased from zero to maximal, the calculated membrane potential increased only by about 6 mV. (*B*) At an external [Ca] = 12.6 mM/l., the "propagated response" fails at a [K] = 16 mM/l. Tension in a strong longitudinal AC field (16 V/cm.), however, increases by gradual depolarization with excess [K], up to a value of 26 mM/l., then declines with further increase in the $[K]_0$. The results of a series of such experiments, performed at different external [Ca], are plotted. Note that by correcting for the effect of excess [Ca] on the membrane potential it can be shown that at a membrane potential of, for example, 40 mV, tension in the K-depolarized muscle is a function of the [Ca].

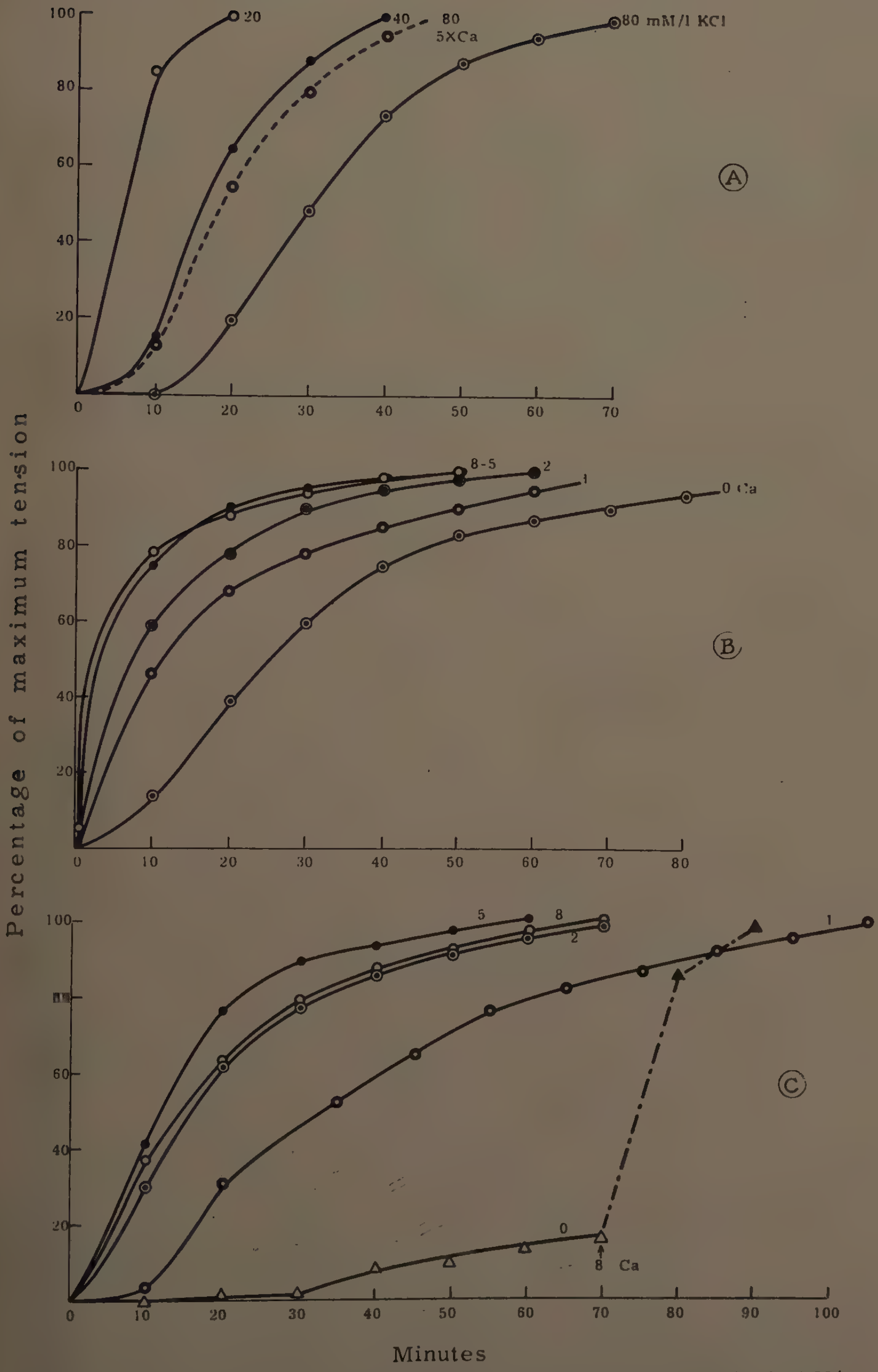

FIGURE 7. Turtle retractor penis muscle, 20° C. Stimulus: longitudinal AC, 1 V/cm., 60 cps. (*A*) The muscle is depolarized by excess [K] = 20, 40 and 80 mM/l., for 30 min. in Ca-free Ringer's solution in order to show that recovery from such treatment in normal Ringer's solution is a function of the $[K]_0$ during depolarization. Note that excess [Ca] during recovery increases its rate. (*B*) Recovery from treatment with excess $[K]_0$ = 80 mM/l. is a function of the $[Ca]_0$ during K-depolarization. (*C*) Recovery from treatment with Ca-free Ringer's solution, containing excess [K] of 80 mM/l., is a function of the $[Ca]_0$ during recovery. Note that in Ca-free Ringer's solution recovery is not only delayed, but is also partial. An increase in the $[Ca]_0$ results in sudden recovery.

perature (for example, 10° C.) returns more quickly than its propagated response (FIGURE 8*a* and *b*), and that the rate of recovery of the latter is a function of the excess $[\text{Ca}]_0$ (FIGURE 8*c*). It appears that the readsorption of Ca is strongly dependent on temperature and on the Ca gradient. Furthermore, muscle can be transiently uncoupled by delaying the readsorption of its labile Ca, as by recovering it in the cold.

These observations substantiate the concept that depolarization and myoplasmic activity are not linked directly, but are connected by at least one intermediate step in which Ca is involved. Muscle can be uncoupled reversibly by removing some of its labile Ca, whereas an uncoupled muscle can be recoupled by treatment with excess Ca. The extent of activation, "threshold," "critical potential," "accommodation," and propagation may be directly related to and perhaps described in terms of the ratio: bound to ionized Ca. However, it may be necessary to distinguish between the Ca in different compartments, that is, in extracellular space, sarcoplasmic reticulum, and myoplasm, and to learn about the forces that determine their respective concentrations and their equilibria. Already three hormones are known, namely, estrogen, progesterone, and oxytocin (Coutinho and Csapo, 1958), that influence the physical state of Ca in the uterus, and it is significant that these hormones are the most powerful regulators of uterine function.

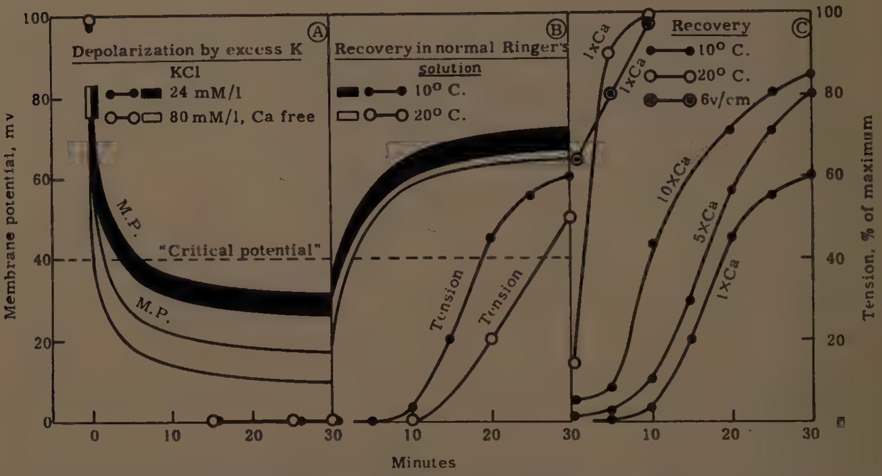

FIGURE 8. Turtle retractor penis, 20° C. Stimulus: longitudinal AC, 1 V/cm., 60 cps (*A*) Two muscles are depolarized by excess-K Ringer's solution for 30 min., one by $[\text{K}]_0 = 24$ mM/l., the other by $[\text{K}]_0 = 80$ mM/l., Ca-free. The membrane potential drops below the "critical" level and the "propagated response" fails. (*B*) When these muscles are recovering in normal Ringer's solution (at 20° C.) from 80 mM/l. [K], or (at 10° C.) from 24 mM/l. [K] treatment, their membrane potential quickly rises above the critical value, but their propagated response reappears only after some delay. Complete recovery of the propagated response requires more than 60 min. (*C*) The rate of recovery at low temperature is increased by excess [Ca]. Although during the first 10 min. the muscle develops no propagated response in normal Ringer's solution when stimulated in a longitudinal AC field of 1 V/cm., it does respond effectively to a stronger field of 6 V/cm.

Another possible approach to the coupling problem is provided by the ingenious myofibril preparation of Natori and Sakai (1958), which enables us to remove the surface layers of a single frog muscle fiber, under oil by microsurgery, and thereby obtain a bundle of "naked" myofibrils about 30 μ in diameter. The ends of the fiber are attached to two platinum electrode plates. Through these electrodes the naked myofibrils can be effectively stimulated by a 3 to 8 V/cm. (AC or DC) longitudinal field (Sakai and Csapo, 1958). Contraction can also be elicited by an anodal current on touching the naked myofibrils with a micropipette or by a silver wire serving as anode. Since shortening is judged by ocular observation only, further experiments with improved and quantitative methods are required before these results can be interpreted safely.

Potassium-depolarized, nonpropagating muscle was useful in showing that current can trigger resting muscle without the necessity of membrane potential change. This preparation, however, failed to provide us with direct evidence for the triggering effect of current in normal muscle, because of its relative insensitivity to current. Turtle muscle, recovering from excess K treatment in normal Ringer's solution, while still nonpropagating, appears to be more similar to normal muscle in its sensitivity to current, however. Such a muscle, when still "uncoupled" due to Ca deficiency or delayed readsorption of Ca at low temperature, seems to have a considerably greater sensitivity to current than a muscle suspended in excess K. Thus, depending on experimental conditions, the sensitivity of muscle to current is different, and it is quite possible that normal muscle, when suddenly and excessively depolarized, is more sensitive to current than in any other condition.

Consequently, it appears justifiable to view depolarization not as the immediate cause, but only as a first step in the series of events leading to myoplasmic activity; to study further the relationship between depolarization and Ca movement, the location of Ca in different muscle structures, the reaction in which Ca seems to play a key role and, perhaps, subsequent steps that precede and participate in the triggering of the contractile proteins.

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Part III. Mechanochemistry

THE ENERGY SUPPLY FOR MUSCLE CONTRACTION*

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In 1950 A. V. Hill reminded biochemists that "No certain evidence exists that any of the chemical changes at present known, or believed, to take place as the result of muscular activity, occur otherwise than in recovery . . . . There is no direct evidence that the breakdown of adenosine triphosphate, for example, ever occurs in living muscle except under conditions of extreme fatigue verging on rigor." However, adenosine triphosphate is of crucial importance for many types of biochemical reactions, and is widely believed to be the direct energy source for working muscle. Thus, Krebs and Kornberg (1957) stated: "It is well established that ATP plays a key role as an energy transmitter in muscular contraction . . . ."

It is believed that adenosine triphosphate is split to adenosine diphosphate during contraction, but is rapidly resynthesized during recovery by creatine phosphate. Creatine phosphate is later resynthesized (via adenosine triphosphate) by well-known biochemical pathways during anaerobic glycolysis and oxidative metabolism, which are known to canalize energy into adenosine triphosphate. It is instructive to look at the evidence for the belief that the energy is transferred to the contractile mechanism by adenosine triphosphate itself.

A century ago there was thought to be in muscle a giant molecule, inogen, that comprised all the protoplasm. On stimulation, it broke down to take up oxygen, give off carbon dioxide, and contract. This theory was disproved by Fletcher and Hopkins (1907), who separated the oxygen uptake from the carbon dioxide output and showed that lactic acid was produced during fatigue and in rigor. Parnas and Wagner (1914) found that this lactic acid came from muscle glycogen and, as Meyerhof (1920) confirmed, this glycolytic reaction constitutes the major over-all energy supply during normal anaerobic contractions. Careful measurements of the initial heat produced in muscle (see A. V. Hill, 1928) indicated that the chemical reactions directly involved in muscle contraction are nonoxidative, while D. K. Hill (1940) proved that the increased oxygen consumption by muscle begins only after the mechanical activity in a twitch is over.

This "lactic acid" theory of muscle contraction was accepted for twenty years until Lundsgaard (1930*a*) showed that, in the presence of iodoacetate, muscles could contract without producing lactic acid. In the meantime, creatine phosphate had been found in muscle and had been shown to decrease during contraction and to be resynthesized during the subsequent recovery period (Eggleton and Eggleton, 1927; Fiske and SubbaRow, 1929; Nachmansohn,

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1928). For a series of twitches in the iodoacetate-treated muscles Lundsgaard (1930*a* and *b*) found a breakdown of creatine phosphate of approximately 0.5 μmole/gm. muscle/twitch, enough to supply the energy for the work done and the heat produced during the twitches. However, he wrote in 1934: "It must be assumed that muscle poisoned with iodoacetic acid utilizes (per gram) an amount of energy equivalent to that in 0.30 to 0.50 mg. P_2O_5 as phosphagen (creatine phosphate) over and above the energy which is available from the phosphagen in the muscle. A breakdown of (organic) pyrophosphate (adenosine triphosphate) could be observed only during excessive fatigue and the development of rigor. The energy utilized in excess of that which came from the phosphagen cannot come from a splitting of (organic) pyrophosphate." * This important statement seems to have been buried in the literature for the last 25 years.

The apparent significance of creatine phosphate was much reduced following the work of Lohmann (1934), who found that the cofactor for the breakdown of creatine phosphate in dialyzed cell-free muscle extracts was adenosine diphosphate. Thus, although adenosine triphosphate remained unchanged (except in extreme fatigue), it was generally accepted that dephosphorylation of adenosine triphosphate was the essential reaction for the provision of energy for contraction (see Needham, 1956).

This was all the direct evidence with intact muscle for adenosine triphosphate and, in 1956, even the tenuous support provided by the Lohmann reaction was removed by Cori *et al.* (1956). These investigators found in skeletal muscle a direct route to creatine phosphate and back from 1:3-diphosphoglyceric acid that did not involve adenosine triphosphate. Thus, creatine phosphate can break down by reaction with 3-phosphoglyceric acid, and the observation of its breakdown can no longer be accepted as evidence that it must have reacted with adenosine diphosphate.

All the other evidence for adenosine triphosphate was based on the finding that myosin is an adenosinetriphosphatase (Engelhardt and Ljubimowa, 1939) and on the well-known work with actomyosin threads and glycerinated muscle fiber models associated with the schools of A. Szent-Györgyi (1953), H. H. Weber (1958), and others. Many have found the argument by analogy from these models convincing, but others have had serious doubts.

There was thus a clear need to obtain more results with intact muscle and, in 1951, A. Fleckenstein came to Sheffield and planned the experiments that have been carried out subsequently at the universities of Pennsylvania, of Sheffield and Oxford in England, and of Heidelberg and Freiburg in Germany.

An important relation is that in a muscle doing 125 gm.-cm. external work per gram of muscle in a single contraction (a low value), a "high-energy" compound releasing 12,000 cal. per gram-mole (a high value) with 100 per cent efficiency in energy transduction would be decreased by approximately 0.25 μmole per gram of muscle. Under actual conditions where heat is produced, a decrease of 0.5 or more μmole per gram of muscle might be expected. The analytical methods must therefore be sensitive enough to detect such changes if they occur.

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* My translation (R. E. D.).

In the early experiments the slow, thin rectus abdominis muscle of female frogs was used. Contraction was induced by electric stimulation, potassium chloride, acetylcholine, nicotine, succinylcholine, or caffeine. In all cases, at 20° C. there was a sufficient breakdown of creatine phosphate to account for the work done, although adenosine tri- and diphosphates certainly remained unchanged, except with the caffeine contracture, when inosine monophosphate was formed from the adenine nucleotides. Experiments at 20° C. were not ideal, because the control muscles contracted somewhat on freezing in liquid nitrogen. However, it was found that at 0° C. the control muscles froze without any change in length and, at this temperature, the electrically stimulated muscles contracted without decrease in adenosine triphosphate or creatine phosphate or increase in adenosine diphosphate, although the contractions produced by the pharmacological agents at 0° C. were accompanied by large decreases in creatine phosphate.

Since the amount of creatine phosphate in the muscle was large compared with the expected decrease, attempts were made to find conditions where the expected change in the concentration of creatine would be greater on a percentage basis. Preincubation with 2:4 dinitrophenol was found to reduce the creatine phosphate content of the tissue from about 11 μmoles/gm. to 0.6 μmole/gm., but the muscles contracted vigorously upon electric stimulation even at 0° C. Under these conditions the expected decrease in creatine phosphate content was 50 per cent or more, yet again there was no significant change in adenosine tri- and diphosphates and in creatine phosphate, although a change of the expected amount would have been readily detected. The low level of creatine phosphate rendered any rephosphorylation of adenosine diphosphate from this source thermodynamically highly improbable.

There was the possibility that creatine phosphate or adenosine triphosphate was resynthesized at the expense of respiration or glycolysis during the duration of the contractions (1.3 to 3.5 sec.) at 0° C.; therefore the rate of recovery of creatine phosphate after electric stimulation for 1 min. was measured. Resynthesis was at least 30 times slower than required for the nondisappearance of creatine phosphate. A similar large discrepancy was obtained from calculations based on the rates of respiration and/or glycolysis of the stimulated muscle and the maximum possible values for the efficiency of oxidative or anaerobic phosphorylation.

These experiments thus show that striated muscle can contract at least once without net fission of energetically equivalent quantities of adenosine triphosphate or creatine phosphate (Fleckenstein, Janke, Lechner, and Bauer, 1954; Fleckenstein, Janke, Davies, and Krebs, 1954). Despite an earlier claim to have observed a breakdown of adenosine triphosphate in a single twitch of a frog muscle (Mommaerts and Rupp, 1951), a conclusion similar to this was reached by Mommaerts (1954, 1955) in experiments with turtle leg muscles at 0° C., in which measurements were made of adenosine tri-, di- and monophosphate, creatine, creatinine, and pyruvate during contraction and relaxation.

In 1953 Munch-Petersen reported an increase in the amount of adenosine diphosphate in cold turtle muscles during a single twitch; but, as Mommaerts (1955) has pointed out and as was realized by Munch-Petersen, the reported differences are of the order of only 0.06 μmole/gm. muscle and are thus too small to provide all the necessary energy. Lange (1955*a*, *b* and *c*) reported decreases in adenosine triphosphate and creatine phosphate and increases in adenosine diphosphate in potassium chloride and acetylcholine contractures of frog rectus abdominis muscle. These contractions took several seconds, and the technique included cutting the muscle from its support and dropping it into a high-speed blender. It is thus doubtful that only the immediate chemical events were being studied in these experiments. Significant amounts of adenosine monophosphate were also found, which was certainly not the case in the experiments of Fleckenstein and his colleagues.

Wajzer *et al.* (1956) found changes in the transmittance of ultraviolet light during a twitch of frog semitendinosus muscle (apparently at room temperature), which they interpreted as due to a deamination of adenine compounds to hypoxanthine compounds. Unfortunately, it was impossible to give even an approximate estimate of the quantities involved, and the changes began well after tension development had begun, so they could hardly have been associated directly with the cause of the contraction. In a later paper (kindly shown to us before publication) Wajzer *et al.* (1958) used a modification of Lange's (1955*a*) technique with a frog leg muscle (rectus internus major) at 0° C. They reported decreases of total adenine nucleotides (average 1.5 μmoles/gm. muscle) and an appearance of approximately 0.5 μmole inosine monophosphate/gm. muscle in a single contraction induced by electric stimulation. The time required to denature the enzymes by the Lange technique and the use of a relatively fast muscle seem to us to be the crucial points. In experiments conducted by ourselves and Fleckenstein and his colleagues, more than 400 pairs of experiments have been carried out; without doubt such a decrease of adenine nucleotides did not occur, nor could have been missed, nor was there a significant appearance of inosine monophosphate except in the case of the caffeine contracture at 20° C. It is, of course, well known that adenosine triphosphate is rapidly broken down and inosine monophosphate rapidly produced in damaged muscle (see Szentkirályi, 1957).

Finally, we must consider the work of Chance and Connelly (1957) and Jöbsis and Chance (1957). These investigators used a highly sensitive quantitative spectrophotometric method for estimating the adenosine diphosphate in the muscle sarcosomes *in situ*, and concluded that only 2 to 3 per cent of the expected value calculated as adenosine diphosphate, or 30 to 60 per cent calculated as inorganic phosphate, was produced in a single twitch, and that no free adenosine diphosphate is released by the muscle contraction until the tension development is largely completed. Calculations of the diffusion time of adenosine diphosphate from unfavorable locations show that it is at least 5 and probably 11 times shorter than the observed lag. However, this calculation was made on the assumption that adenosine diphosphate can diffuse freely within the muscle, which has not yet been proved, and there is the possibility that some of the presumed adenosine diphosphate might have been resynthesized before it reached the sarcosomes. Another point is that the effects found were assumed to be caused by the release of adenosine diphosphate or of inorganic phosphate only because thus far no other compounds are known that produce the effect studied. This evidence is therefore acceptable to exclude adenosine diphosphate, but not to prove that the effect found was in fact due to adenosine diphosphate.

On the basis of these rather confused and contradictory results, our view is still that, under some conditions, contraction of muscle can occur without net fission of adenosine triphosphate or creatine phosphate, although we accept the fact that net changes of creatine phosphate occur after a single normal twitch or during a series of normal twitches.

There remains the possibility that there is an exceedingly rapid turnover of the phosphorus of the adenosine triphosphate, and many experiments with radioactive phosphorus have been directed toward this problem (see Needham, 1956 for the early work). In the most recent experiments the specific radioactivities were measured of, *inter alia*, the α-, β-, and γ-phosphorus atoms of adenosine triphosphate and of creatine phosphate during single twitches and prolonged tetani (Fleckenstein *et al.*, 1956*a* and *b*; Dixon and Sacks, 1958). The results give no support to the view that there is an increased turnover of adenosine triphosphate during muscular activity.

However, there are difficulties in interpretation. It may well be that the organic phosphates of muscle are functionally compartmentalized, that is, that only a small fraction of the adenosine triphosphate is involved in contraction. This could account for the observed failure of isotopic equilibrium to be reached between the γ (terminal) phosphate of the total adenosine triphosphate and the creatine phosphate, despite a major breakdown and resynthesis of the creatine phosphate during and after a tetanus. This means that the results of these isotopic experiments cannot be used to rule out a role for adenosine triphosphate in muscle contraction.

This situation was clearly unsatisfactory; therefore, efforts were made to investigate possible changes in other compounds. In experiments done partly in collaboration with M. L. Bobb at Oxford University it was found that the chromatographic procedures previously developed at Sheffield and Heidelberg universities for the isolation and measurement of the several fractions of the acid-soluble phosphorus of the muscle would have included with the various adenine nucleotides all of any nucleotides of guanine, cytosine, and hypoxanthine that might have been present. These compounds are therefore also excluded as the energy source for contraction.

It had been noted that with both frogs and toads there was a discrepancy between the amount of total phosphorus put on the paper and that recovered after development of the chromatogram. This missing phosphorus was found in two spots that reacted with ninhydrin after prolonged heating, but which failed to react with the usual acid-molybdate spray for phosphate even after hydrolysis sufficiently severe to fragment the paper. Further work showed that these compounds did not change during contraction, failed to become radioactive at any significant rate after injection of radioactive phosphate, and were either absent or not present in sufficiently large quantities in muscles from mammals or birds to provide sufficient energy for contraction. They were not investigated further, and are probably phosphodiesters of the type found by Roberts and Lowe (1954).

The work was now transferred to the University of Pennsylvania, where a search was made for very labile phosphorus and other compounds that might change on contraction. A reinvestigation was made of the question of the release of inorganic phosphate during a single twitch. The previous experiments of Fleckenstein, Janke, Davies, and Krebs (1954) had suggested such a release, but the methods used had not been designed to answer that question, and the results were not sound enough statistically.

The very slow, thin, paired, striated retractor penis muscles of turtles were selected for some of these experiments. This muscle can be frozen in melting dichlorodifluoromethane (-155° C.) in the relaxed state without change of length, and measurements of the longitudinal electric conductivity showed that the center of the muscle was frozen in less than one fifth of the time needed for a normal contraction at 0° C. Thus, there was no time for a possible isometric contraction of the internal parts of the control muscle to occur during freezing that might have vitiated the results. Inorganic phosphate was measured at 0° C. or −36° C. in methanol-water extracts of the powdered muscles made at −36° C. by methods similar to those recently described by Wahler and Wollenberger (1958). The method is quite complex and is still being improved, but the results from the first short preliminary series of pairs show that in the resting muscles the "true" inorganic phosphate was 1.9 and that in the contracted muscles it was 2.4 μmoles/gm. The standard error of the difference between the means (0.5) was ±0.11. These concentrations are much lower than those found after extraction with trichloroacetic acid at 0° C. (approximately 12 μmoles/gm.) and are comparable with those reported recently for heart muscle by Wollenberger *et al.* (1958). These low values for the "true" inorganic phosphate mean that the actual free energy released from the decomposition of "high-energy" phosphate compounds in the muscle must be somewhat higher than was previously believed (see Burton, 1958).

In recent years the possible role of carnosine phosphates in muscle contraction has received attention (Severin *et al.*, 1948) and, as Goodall (1956) has pointed out, its nondetection so far may have been because it is so much more acid-labile than is creatine phosphate. Experiments were therefore carried out in which the turtle retractor penis muscles were preincubated for several hours in a physiological salt solution containing radioactive inorganic phosphate, stimulated electrically and then allowed to recover. This causes a chemical turnover of all phosphate compounds involved in muscle contraction and ensures a close approach to equality in the specific radioactivities of these labile phosphates. A mixture of synthetic carnosine di- and monophosphates was added to the frozen, powdered muscle and then reisolated by chemical and chromatographic tchniques.

The first isolation method used showed in 11 of 11 experiments that the radio-activity associated with the carnosine phosphate on the chromatogram disappeared either completely or almost completely during a single twitch. The amounts of this material found on the chromatograms, based on a comparison with the radioactivity of the creatine phosphate present, were in the range 0.3 to 1 μmole/gm. muscle (FIGURE 1); but the compound was apparently even more acid-labile than the carnosine phosphates. The use of another solvent for developing the chromatograms showed that in this case the added carnosine phosphates had no associated radioactivity and therefore did not occur in this muscle (see FIGURE 2 and Cain *et al.*, 1958). The labile phosphorus compound is not separated from creatine phosphate in this solvent.

These investigations have only recently begun to come to fruition; clearly, much remains to be done. However, they support the view that inorganic phosphate is in fact released during a single muscular contraction in the expected amount and is formed from a highly labile phosphate compound that has been isolated, but that remains to be identified.

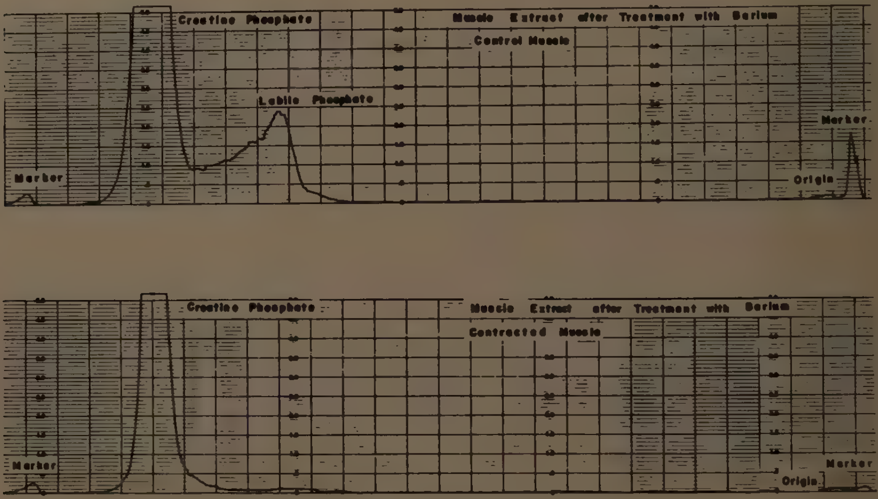

FIGURE 1. Distribution of radioactive phosphorus in a chromatogram of barium-treated muscle extract. Note the virtually complete disappearance in the contracted muscle of the area called "labile phosphate" in the control muscle. Full-scale deflection of the scanner at the sensitivity used for this graph was 3000 c.p.m.

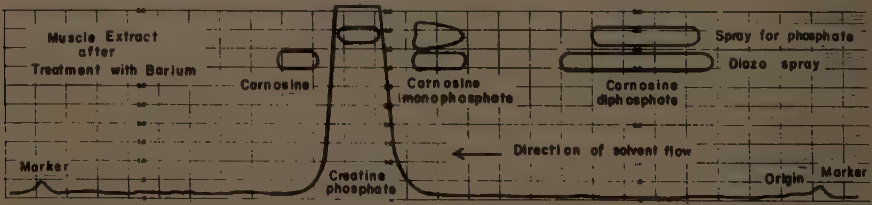

FIGURE 2. Distribution of radioactive phosphorus in a chromatogram of barium-treated muscle extract. In the solvent used for this chromatogram of the extract of resting muscle, the "labile phosphate" had the same R_F as the creatine phosphate. Carnosine phosphates had been added to the powdered frozen muscle before extraction, but had no associated radioactivity after isolation on the chromatogram.

Whether this compound is required to maintain relaxation or provides the energy for a single contraction or acts directly, indirectly, or not at all, involves questions that remain to be investigated.

Meanwhile, these results explain why we remain somewhat skeptical that adenosine triphosphate itself is acceptable as the final energy donor for muscle contraction, and take our stand with William of Ockham (Fourteenth Century) "*Entia non sunt multiplicanda praeter necessitatem.*"*

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* "Entities must not be unnecessarily multiplied."

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THE RESPONSE OF MITOCHONDRIA TO MUSCULAR CONTRACTION

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Introduction

In this monograph controversial points hinge, as they always will, upon whether the biological system has changed its physicochemical properties in the transition from its physiological state to its analysis by physical or chemical methods. For example, electron microscopy requires fixation procedures, chemical analyses require cell destruction, and some sensitive electric measurements require rupture of the cell membrane. In spite of the important contributions made by such methods, it is occasionally necessary to employ for control experiments methods that, we may agree, cause no measurable change in the physiological state of the cell. Examples of methods in the "harmless" category are the measurement of temperature and of light absorption, and some measurements of electric potentials. These three methods are, in general, nonspecific, since they do not refer the measurement to a particular location of the cell or to a particular chemical reaction in the cell. However, remarkable progress has been made in microelectrodes for localizing potential. Heat measurement, as used by A. V. Hill,[1, 2] suffers from a lack of localization and from a confusion as to the source of heat; Hill reviewed at least four kinds of heat that could be responsible for the observed effects.[3] In fact, it is only by time separation that some of these heats can be obtained independent of others. Optical methods would appear to suffer similarly from lack of specificity. For example, light-scattering and diffraction measurements carried out by a number of investigators, most recently by William Sleator,[4] give chiefly physical evidence of structural changes in the muscle. Recently, however, special spectrophotometric techniques have been developed to minimize the light-scattering and diffraction effects that occur during a muscle twitch and to maximize the small absorbancy changes due to intracellular enzymes.[5] Thus we have a new tool for measuring the metabolic response of muscle to contractions.

Optical methods provide two means for the localization of intracellular events. First, a degree of localization is obtained by spectrophotometric recordings of changes in a cell constituent known to be localized in a regular fashion near a characteristic repetitive structure of the muscle. The location of mitochondria near the I bands of muscle enables us to refer measurements of cytochrome and certain types of pyridine nucleotide changes in excised muscle tissue to this region. Second, localization may be provided by microspectrophotometry,[6] which has recently been perfected to the degree that cytochromes in mitochondrial aggregates may be measured fairly accurately.[7]

Particularly needed is a method that will indicate changes of intracellular adenosine diphosphate (ADP) and phosphate concentrations within the living, intact muscle a short time after the beginning of the muscular contraction. There is currently a great interest in determining what substances are broken down during the muscle twitch, for these are the data on which any theory on the chemical aspects of the muscular contraction must be built. As explained by Davies *et al.* elsewhere in this monograph, all chemical assays must be done upon the disrupted cell, and any differences between control and experimental muscles involve the assumption that a stimulated muscle behaves in the same way as the control muscle throughout the disintegration procedure. This may or may not be the case. Upon this assumption hang such chemical analyses of muscle.

Respiratory Control

The feasibility of using light-absorption changes in the living muscle to interpret changes of ADP or phosphate in the early phases of muscle twitch depends on a well-known but little-understood phenomenon characteristic of many muscles: namely, that of respiratory control. Respiratory control may be defined as the ability of living tissue to accelerate its respiratory rate during activity and to return to a quiescent state characterized by a slow respiratory rate when metabolic activity has led to a restitution of the chemicals expended during activity. Many aspects of respiratory control have recently been summarized by Lardy.[8] Some quantitative aspects of respiratory control can be determined by studies of isolated mitochondria. These will be reviewed briefly.

Range of respiratory control ratios. For isolated mitochondria it is important to define respiratory control ratio as the quotient of the rate obtained in the presence of added ADP to that obtained following the expenditure of added ADP.[9, 10] The reason for setting up the criterion in this form is that loosely coupled mitochondria contain ATPases that are activated by the ATP (adenosine triphosphate) formed upon the phosphorylation of added ADP.

Some examples of maximal respiratory control ratios are given in FIGURES 1 and 2, which contain recordings made by means of a vibrating platinum electrode. In heart muscle mitochondria suspended in a sucrose versene medium (FIGURE 1), an acceleration of respiration upon ADP addition takes place, but upon ADP exhaustion respiration comes to a halt; the ratio of the rates exceeds 20:1. One of the highest values of respiratory control ratios observed by us in isolated mitochondria is given in FIGURE 2, where guinea pig liver mitochondria are represented. This particular curve indicates a ratio of 65:1.

A summary of respiratory control data is given in TABLE 1.[11] Here it may be seen that many types of tissues provide mitochondria that exhibit respiratory control ratios in excess of 10:1. However, some cells are apparently so resistant to rupture that mitochondria prepared from them may be damaged. This may explain the lower ratios obtained with turtle liver, toad heart, ascites tumor cells and, in particular, mitochondria isolated from yeast cells for which respiratory control has not yet been demonstrated (Chance and Nossal, unpublished data). It is very probable that a high respiratory control ratio is characteristic of intact mitochondria. The extent to which this control ratio can be demonstrated in the intact cell depends upon the extent to which the work done by the cell can be controlled. It is obvious that skeletal muscle is an excellent tissue for the demonstration of respiratory control *in vivo*.

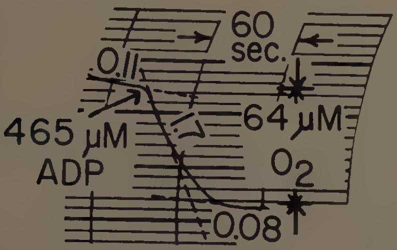

FIGURE 1. Example of respiratory control in a heart muscle mitochondrial suspension (experiment 615).

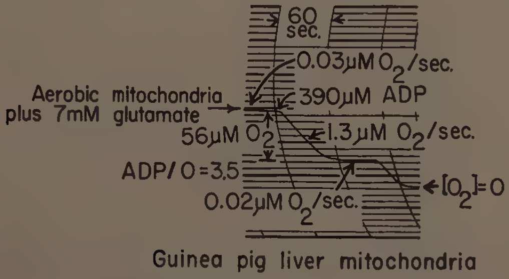

FIGURE 2. Example of respiratory control in a guinea pig liver mitochondrial suspension (experiment 372).

TABLE 1*

OPTIMAL VALUES OF RESPIRATORY CONTROL RATIOS IN ISOLATED MITOCHONDRIA

Source	Medium	Substrate	Respiratory control ratio	Expt.
Rat liver	Salts	β-Hydroxybutyrate	>20 (38)	337[12]
Rat heart	Sucrose-versene	α-Ketoglutarate	>20 (21)	615[9]
Guinea pig liver	Salts	Glutamate	>20 (65)	372[12]
Guinea pig kidney	Tris-sucrose	Glutamate	17	644†
Turtle liver	Salts	Glutamate	8	641‡
Toad heart	Sucrose-versene	Succinate	5	815§
Ascites tumor cells	Salts	Succinate	6	662‖

* Reproduced by permission of the Ciba Foundation, London, England.[11]
† B. Chance and G. Hollinger (in preparation).
‡ Experiments done in collaboration with F. Jöbsis.
§ Experiments done in collaboration with J. Ramirez.
‖ B. Chance and B. Hess (in preparation).

ADP and phosphate affinity of isolated mitochondria. Measurements of the stimulation of respiratory rate in response to additions of small concentrations of ADP give, by definition, the ADP affinity of the system. FIGURE 3 indicates the effect of ADP concentration upon respiratory rate for guinea pig liver mitochondria with succinate as substrate; 20 μM ADP gives half-maximal respiratory stimulation.*

* All concentrations are given in moles per liter.

It has been found that spectroscopic responses accompany these respiratory changes and that measurement of the changes in the oxidation-reduction level of any one of several respiratory carriers allows a much more sensitive determination of ADP affinity. FIGURE 4 gives an example of the addition of small amounts of ADP to azide-treated mitochondria. These mitochondria respond with abrupt changes of oxidation-reduction level (reductions of cytochrome *a*) that are followed by an oxidation when the added ADP has been expended. Larger amounts of ADP cause a greater reduction in the steady state and a longer duration of the cycle, with a correspondingly longer time required to expend the added ADP. In fact, the area under the curve is proportional to the ADP concentration. These records are examples of the basic principles of the spectroscopic method for the measurement of changes of intracellular ADP concentrations. Four aspects of the indication may be pointed out here:

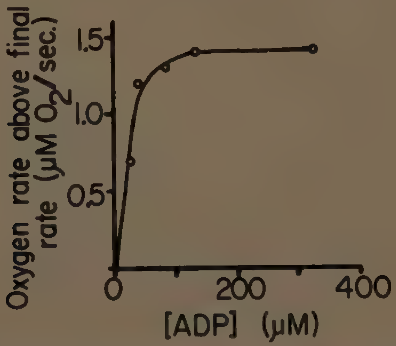

FIGURE 3. Effect of ADP concentration upon the respiratory rate of a rat liver mitochondrial suspension (experiment 379c).[10] Reprinted by permission from *The Journal of Biological Chemistry.*

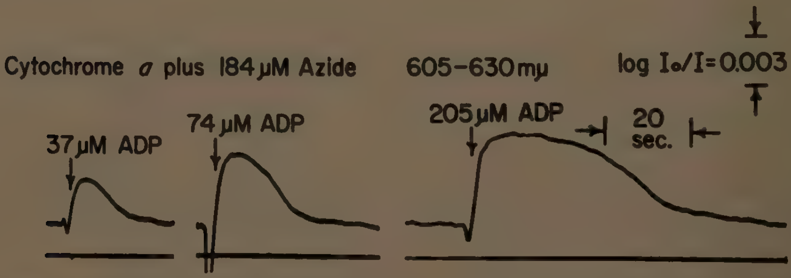

FIGURE 4. Spectroscopic response of cytochrome *a* to additions of small concentrations of ADP. Suspension of azide-treated rat liver mitochondria, spectrophotometric recordings with the double-beam apparatus (experiment 388b).[12] Reprinted by permission from *The Journal of Biological Chemistry.*

(1) The indication is rapid; at room temperature the steady state is reached in a fraction of a second.

(2) The measure is quantitative; the more ADP added, the greater the absorbancy change.

(3) The area under the curve also gives a measure of the amount of ADP turned over.

(4) The indication is highly specific for ADP; no other known nucleotide causes a similar response.

Similar experiments have been carried out with inorganic phosphate, for which the results are qualitatively similar: phosphate addition causes a change in the steady-state levels of the respiratory carriers. Quantitatively, there are two important differences:

(1) The affinity for phosphate is roughly one fiftieth that for ADP at *p*H = 7.4.

(2) The nature of the cycle differs: the speed with which the steady state is established is somewhat slower and, since a given amount of phosphate causes considerably less activation of respiration than the same amount of ADP, the cycles are prolonged and do not show the abrupt termination indicated by the ADP cycles. In summary, the response of the mitochondria to phosphate is sluggish and that to ADP rapid.

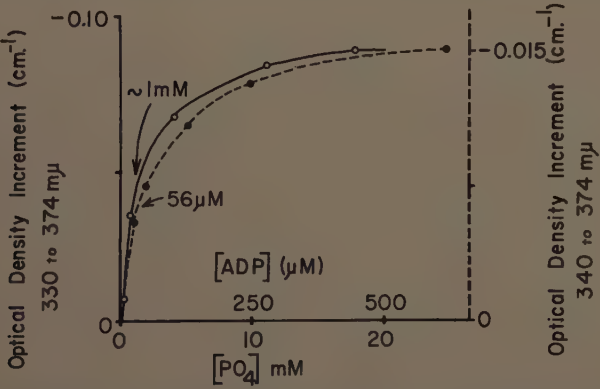

FIGURE 5. Comparison of titrations of the response of intramitochondrial pyridine nucleotide to ADP and phosphate. Note different scales of the abscissa (experiments 299b, 463). Reprinted by permission from *Nature*.[5]

A comparison of the titration of the pyridine nucleotide of isolated mitochondria with ADP and phosphate is shown in FIGURE 5.

Methods

Now that we have presented a method that can be applied to the measurement of changes in ADP or phosphate concentrations within living tissues, it is worthwhile to review the experimental methods for such measurements and to indicate their sensitivity.

The wave lengths at which cytochromes can be observed to change their oxidation-reduction state are indicated by the peaks of the absorption bands that appear when an aerobic muscle is treated with nitrogen. The muscle may be stimulated to speed the attainment of anaerobiosis. Such absorption bands are shown in FIGURE 6 for frog sartorius muscle. This clear recording, obtained in our laboratory by A. Weber, shows absorption bands caused by cytochromes, flavoproteins, and pyridine nucleotides.

The apparatus that we have found most suitable for measuring the changes of intensity of these absorption bands does not measure at one wave length, as is the usual case with the spectrophotometer, but measures instead the difference of absorption between any two wave lengths. There are a number of advantages to this, the most obvious being that it is possible to select a pair of wave lengths that will measure one cytochrome component largely to the exclusion of an interfering component. Such wave lengths are indicated in TABLE 2:[13] the measuring wave lengths appear in the upper line, the reference wave lengths in the lower. A consideration of the difference spectra of FIGURE 6, together with experimental studies with inhibitors that selectively affect one or two components of the respiratory chain, reveals that differences of intensity recorded at these wave lengths correspond almost exclusively to the component named. The only interference that causes some concern in the intact tissue is that involved in small changes in cytochrome a_3 and large changes of flavoprotein.

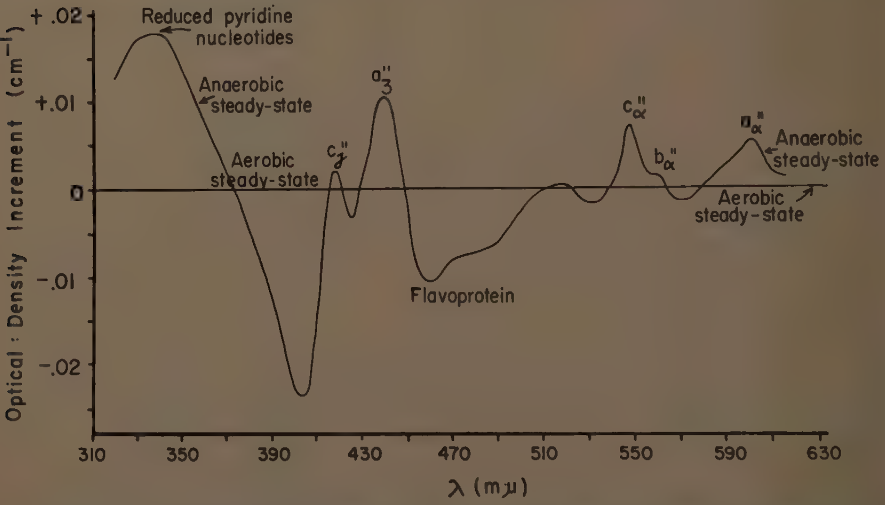

FIGURE 6. The spectrum corresponding to the difference between the reduced and oxidized forms of the cytochrome of a 1 mm.-thick frog sartorius muscle. Experimental data by courtesy of A. Weber.

Although the autofluorescence of intact tissues has been studied for a number of years and has been attributed to mitochondria,[14] we have found the blue fluorescence of the living tissue to be caused chiefly by the reduced pyridine nucleotide of the mitochondria.[15, 16] Furthermore, following the results obtained in the laboratories of Theorell[17] and Duysens,[18] Baltscheffsky and I have shown that this corresponds to a bound type of pyridine nucleotide with a maximum at 443 mμ.[19] The method appears to be equally as sensitive as the spectrophotometric one, and has the considerable advantage that light transmission through the sample is not required; fluorescence may be excited by oblique illumination and the emitted light may be measured readily with a closely placed photocell.[18, 20] The technique would therefore have the advantage of observing changes in the steady state of pyridine nucleotide in the surface layer of a thick muscle. It may also have the further advantage, which I must admit we have not yet investigated, that hemoglobin would probably not interfere. Finally, there is the advantage that the fluorescence signal emitted at 440 mμ is caused primarily by reduced pyridine nucleotide; other components do not have interfering emission bands in this region. However, a difficulty exists in that, with measurements of pyridine nucleotide, the sum of cytoplasmic and mitochondrial pyridine nucleotides is measured. At reduced temperatures, the dominant response to contraction is found to be that of mitochondrial pyridine nucleotide. Microspectrophotometry of intramitochondrial reduced pyridine nucleotide has recently been developed to the point where adequate signals can be obtained from a mitochondrial aggregate,[21] but the method has not yet been applied to muscle.

TABLE 2*

APPROPRIATE WAVE LENGTHS FOR MEASUREMENT OF RESPIRATORY ENZYMES

The Figures in Parentheses Refer to Neutral Reference Wave Lengths Used in Double-Beam Spectrophotometry

O_2 → cytochrome a_3 →	cytochrome a →	cytochrome c →	cytochrome b
445 mμ	605 mμ	550 mμ	564 mμ
(460)	(630)	(541)	(575)
		→ flavoprotein →	reduced pyridine nucleotide → substrate
		465 mμ	340 mμ
		(510)	(374)

* Reproduced by permission from *Science*.[13]

Localization of the Response

It is important to consider whether mitochondria are localized in the proper place in muscle in order to qualify as indicators of the change of ADP concentration caused by muscular contraction. That the cytochrome system is involved in the oxidative recovery process is shown by the cyanide and carbon-monoxide sensitivity of the "activity" respiration. Although the concentration of mitochondria may vary from muscle to muscle, the spectrophotometric method is used in such a way that the response is evaluated in terms of those mitochondria affected by ADP. Thus, mitochondria located near the cell nucleus or in portions of the muscle in which the ADP concentration might not change during the twitch do not adversely affect the recordings. Electron micrographs show clearly that the muscle mitochondria are interwoven with the myofibrils. Not only is there such intimate contact that the mitochondria themselves appear to be deformed, but there probably occurs during muscular contraction a rolling or sliding motion that causes even more intimate contact with the contractile elements and the mitochondria. It is not unreasonable to speculate that the hypothetical pores in the endoplasmic reticulum may provide a very ready exchange of chemicals between the sarcoplasm and the mitochondria. Such an intimacy of contact combined with the possibility of facilitated diffusion due to relative motion and perhaps even pore action suggests that diffusibility of ADP from the myofibril to the mitochondria may not be a limiting factor.

The mitochondria are sharply localized centers of enzyme activity and the actual concentration of cytochrome at the site of optical measurement is very high. At a particular mitochondrion, the preponderance of activity for respiratory resynthesis to other activities is probably very large. By way of analogy, it is useful to consider that the mitochondria act as indicators of the changes of ADP concentrations strategically located in some muscles near the I bands.

Choice of tissue. A muscle approximately 1 mm. thick and 2 to 3 cm. long that is free of myoglobin and that can be readily perfused is optimal for the currently available experimental apparatus. At the present time, experimental data on the response of mitochondria to muscular contraction have been obtained in frog sartorius muscle, toad sartorius muscle, turtle coracohyoideus muscle, and in the heart muscle of the frog, toad, and lobster by C. M. Connelly, A. Weber, F. Jöbsis, D. Lubbers, and J. Ramirez.

Muscle holder. One type of muscle holder, developed in collaboration with V. Legallais, has been described elsewhere.[13] In brief, the muscle is held between two thin perforated Lucite plates so that ready access to the oxygenated Ringer's solution is obtained. The optical path in frog sartorius muscle is about 1 mm. long, 4 mm. wide, and 20 mm. or more high. The muscle holder, as used in initial experiments with C. M. Connelly, did not contain methods for measuring tension, but this is now provided in currently used methods developed by F. Jöbsis and V. Legallais. Special muscle holders for use with strips of heart muscle have also been developed (D. Lubbers and J. Ramirez, unpublished data).

Experimental Results

Since reduced pyridine nucleotide has the largest absorption band of the respiratory components, it was logical to begin spectrophotometric experiments on muscle by recording at 340 mμ. In these experiments we used a reference wave length of 386 mμ, since this pair gives a minimal response to oxygenation and deoxygenation of hemoglobin in the few remaining erythrocytes retained by the perfused muscle.

We soon found that lowering the temperature has several advantages. First, the respiratory metabolism is slowed relative to the diffusion of oxygen, and hence the interior of a 1 mm.-thick sartorius muscle can be kept aerobic for a higher number of twitches than at room temperature. As a corollary of this, utilization of ADP or phosphate by the mitochondria is slow, and hence their response to ADP is prolonged. Under these conditions we obtained records of the type shown in FIGURE 7. In this record no indication of tension is given directly, since it was taken at an early stage in the studies. However, independent controls with tension recordings indicate that the small and downward deflections indicated by the arrows are optical disturbances that accurately mark the time of the twitch. Here we find that single twitches of the oxygenated frog sartorius muscle bathed in oxygenated Ringer's solution at 7° C. and stimulated by silver electrodes give a series of stepwise absorbancy changes. For two reasons, this type of response is significant:

(1) It reflects a decrease of absorbancy at 340 mμ with respect to 386 mμ; this is verified by spectra taken at different wave lengths that show the disappearance of a peak at 340 mμ.

(2) The time course of the response indicates that the utilization of ADP or phosphate is slow compared to the rate of its production. To elaborate on this point, if the mitochondria expend very rapidly the ADP produced in the muscle twitch, then the absorbancy change is small and the "cycle" of oxidation and reduction is very rapid. This does not appear to be the case. If the creatine transphosphorylase or other enzyme systems resynthesize ATP at the same time, there would also be a rapid decay of the ADP level immediately after the twitch. Again, the fact that the optical change is approximately constant for a reasonable time indicates that this reaction is not occurring to an appreciable extent in the time interval recorded. Thus, the kinetics of any process expending ADP other than that under observation must somehow be reflected by the kinetics presented in this record. Within the limits of experimental accuracy there is no such evidence, and mitochondria probably provide the chief process responding to the ADP formed in the muscle twitch under these particular experimental conditions. Further documentation for this possibility is afforded by the high affinity of the respiratory chain for ADP. For example, the mitochondria have at least a tenfold greater affinity for ADP than does the transphosphorylase, although the precise data are not available for the latter under physiological conditions.[22] This reasoning also applies to

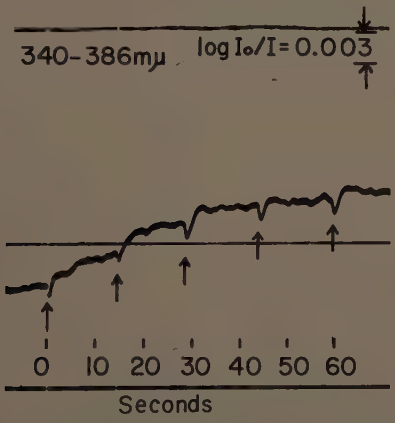

FIGURE 7. Response of frog sartorius muscle at 7° C. to isolated twitches (experiment 301–14).[5]

other methods of resynthesis that may be postulated. In conclusion, we believe that the stepwise nature of the optical response obtainable under certain experimental conditions is very significant.

A preliminary calculation suggests that, although the transphosphorylase activity clearly exceeds the respiratory activity at 38° C.,[22] the relatively greater temperature coefficient of the respiratory activity suggests that the situation would be reversed at the low temperatures (7° C.) at which we usually observe the steady-state changes in cytochromes following a twitch. The fact that rapid cytochrome responses are obtained with both heart and skeletal muscle under appropriate conditions despite their different creatine transphosphorylase activities[23] lends further support to this statement.

If a series of twitches at 1.5/sec. is given, the response is much more rapid, as shown in FIGURE 8. Under these conditions, we can compare the absorbancy change caused by 10 twitches with those obtained by stimulation over longer intervals, as indicated by the arrow (maximum level). From the jogs in the trace of FIGURE 8, one can readily observe that approximately 6 twitches cause a half-maximal absorbancy change. As a control, this record indicates the effect of stimulation of the dinitrophenol-treated muscle in which we obtain not the characteristic disappearance of the 340 mμ band but, instead, a smaller effect in the opposite direction. This control suggests that it is the response of the mitochondrial pyridine nucleotide that dominates under these experimental conditions.

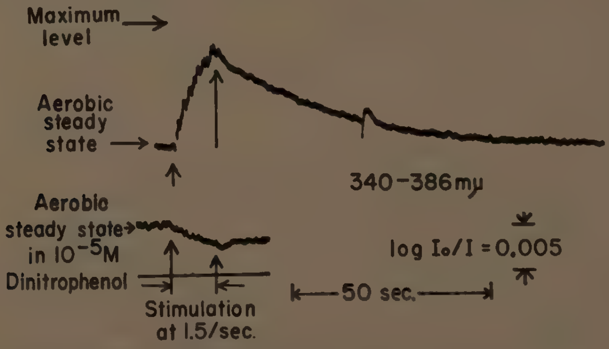

FIGURE 8. Response of frog sartorius muscle at 7° C. to a series of twitches. Insert shows effect of dinitrophenol on the response (experiments 301–3, 301–33).[5]

Calculation of the amount of ADP per twitch. Experiments with two different muscles for various stimulation rates and total twitches are summarized in TABLE 3.[5] It is seen here that, over a stimulation rate of 0.07 to 7.0/sec. and a total number of twitches from 6 to 75, the average number of twitches required to give half-maximal effect is 6.

In studies of mitochondria isolated from rat liver, toad heart, and frog skeletal muscle, we find that about 50 μM ADP causes a half-maximal spectroscopic effect. The ADP released per twitch is then 50/6 μM or 0.009 μmole/gm. The maximum variability of this measurement indicated by TABLE 1 is about 0.001 μmole/gm. The inherent accuracy for a particular observation is considerably better than this, as may be seen from an inspection of experimental records. We feel that there is now evidence for ADP formation under conditions where the chemical methods have failed to find it.

This small value for the ADP release per twitch supports the negative result obtained by chemical analyses in which the amount of change observed by us is approximately equal to the experimental error of the chemical assay method. In other words, the ADP change under the experimental conditions of Mommaerts[24] and of Fleckenstein *et al.*[25] is too small to measure by the chemical procedure, and the more sensitive spectrophotometric method has yielded positive results.

The question of an alternate interpretation of this assay in terms of phosphate must be considered. However, in the light of the extremely high ratios of phosphate to ADP concentrations found in muscle, we suspect that phosphate does not limit the respiratory rate in the resting muscle, and that the observed responses are caused by ADP rather than by phosphate.

Table 3*

Changes of Level of Reduced Pyridine Nucleotide Following Muscle Twitches

Frog Sartorius Muscles, 5 to 7° C. in Oxygenated Ringer's Solution Plus Glucose; Muscle Essentially Unloaded

Experiment no.	292b	292b	292b	301	301	301	301
Record no.	12	13	14	3	4	14	14
Stimulation rate (sec.$^{-1}$)	3	7	1.5	1.5	3	0.07	0.2
Total twitches	75	7	45	15	45	6	27
Maximal oxidation of reduced pyridine nucleotide observed (μM) at given stimulation rate	24	14	23	25	32	11	30
Maximal effect with rapid stimulations (μM)	24	24	24	32	32	32	—
Twitches to give half-maximal effect†	7	6	5	7	7	5	—

* Reproduced by permission from *Nature*.[5]

† We have computed the number of stimulations that have occurred in the time required for the optical change to reach half the "maximal effect" with rapid stimulation. In experiment 301-14 this point was reached by linear extrapolation from the initial increments per twitch. More refined calculations were not justified by the accuracy of the observations.

Discussion

In view of the measured heats of activation and shortening and estimates of the work done by a muscle under these experimental conditions, it is reasonable to expect that between 0.3 and 0.5 μmole/gm. of ADP would have been released per twitch if ATP were broken down in the rising phases of the contraction. Such an increased concentration of ADP would be 10 times the half-saturation value and would have caused the absorbancy change to rise immediately to the maximal value, but this is not the case, as is clearly demonstrated by the experimental records. If we interpret the plateaus of the stepwise response to a series of slow twitches, shown in FIGURE 7, to indicate that ADP utilization during the attainment of the response is negligible, we are faced with the fact that the ADP concentration arriving at the mitochondria is, on a per twitch basis, only a few per cent of that to be expected if ATP were expended directly during the twitch. As an explanation for this discrepancy, it is possible that the muscle is already charged with a high energy compound; this is implicit in the hypotheses of A. F. Huxley and H. H. Weber presented elsewhere in this monograph. According to the theories of these investigators the muscle is charged with a high-energy compound prior to a twitch, so that the first movement of the cross-linked structures does not require the expenditure of ATP. On the other hand, this initial discharge of the crosslinks would lead to a shortening of the sarcomere of only about 400 Å, and hence repetitive recharging and discharging of the crosslinkages would occur in a single contraction, and ADP formation should be observed.

It is now possible to consider both our spectrophotometric observations and Davies' chemical observations in the light of these theories. The important feature of the experimental data presented by Davies elsewhere in these pages is that the store of hypothetical high-energy compound is very nearly completely expended in a single twitch. Inadequate extraction of the compound is unlikely to alter this observation, since it is assumed that the control muscle and the twitched muscle behave similarly in the extraction procedure. It is obvious that, if the muscles behave differently, the assumption of the chemical method (see *Introduction*) is violated, and therefore none of the chemical data is significant. Thus the muscle must depend upon other high-energy stores for twitches subsequent to the first one. This change-over from the endogenous supply to the external supply would be expected to have a marked effect upon the amount of ADP found in the muscle mitochondria, and the increments of absorbancy following a twitch should be small or zero for the first twitch and large for the second and subsequent twitches. As clearly illustrated by FIGURE 7, this is not the case; the ADP appears to rise in steps of nearly equal amplitude for single isolated twitches. In fact, if an explanation of our data is based upon the postulated high-energy intermediates, it is apparent that the energy store must be sufficient to tide the muscle over for not one but for several twitches, and that the amount of this store must therefore be in excess of a few micromoles per gram. Thus the optical measurements suggest that any high-energy store postulated as an explanation of the small concentration of ADP arriving at the mitochondria following a twitch must be of considerable magnitude and capable of tiding the muscle over for a few twitches.

Summary

A sensitive optical method has been used to detect specific chemical reactions in the muscle following a twitch. The method is of high sensitivity and, in terms of change of adenosine diphosphate concentration, will record 0.001 μmole/gm. The method has been applied to the question of the amount of ADP formed following the muscle twitch. This is found to be a few per cent of the full amount expected, and various theories involving the activation of crosslinkages between actin and myosin and of tightly bound high-energy reservoirs in the muscle are considered as explanations for this discrepancy.

It is apparent from the optical data that any high-energy store postulated as an explanation of the small amount of ADP formed following the isolated

twitches in a frog's sartorius muscle must be sufficient to sustain the muscle for not one but for several twitches.

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THE RELAXING FACTOR OF MUSCLE*

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It seems that at the present time we are at a stage in the history of muscle physiology and biochemistry when, as has happened several times in the past, interest is shifting to new aspects, new concepts emerge, and old phenomena are being looked upon from a new point of view. Elsewhere in this monograph Davies *et al.* mention a possible new source of energy for muscle contraction.[3] Whether this is the ultimate source or whether it acts as a phosphagen through adenosine triphosphate (ATP) is an open question at the moment; H. E. Huxley[4] has described the so-called sliding mechanism of contraction, which would replace that based on coiling of protein filaments; Weber's[5] presentation made it clear that the key of the contraction-relaxation cycle of muscle might have to be sought in the relaxation phase.

It is this aspect to which I shall address myself. In the past, perhaps, we have been unduly influenced by our experience with various machines, including our automobiles, where control is exercised through some sort of accelerator. The problem of chemicomechanical coupling in muscle may require a different approach, envisaging a decelerator rather than an accelerator, and this decelerator might be found in the relaxing mechanism. Thus, stimulation would really mean inhibition of the relaxing system.

The current interest in the relaxation phase of the muscle cycle owes its inception to the observation of Marsh in 1951[6] that a fresh suspension of myofibrils shows a great reluctance to settle out on centrifugation. Marsh was able to show that this was due to a swelling of the fibrils in the presence of ATP and an unknown factor present in a fresh muscle extract.[7] This process was accompanied by an inhibition of the splitting of ATP by the fibrils. Bendall,[8, 9] somewhat later, showed that the muscle extract used by Marsh also produced a marked lengthening of loaded glycerinated fiber bundles that have previously been shortened under the influence of ATP. Bendall introduced the term Marsh factor[8] for the principle presumably present in the extract, and soon various names were adopted for whatever was present therein. Hasselbach and Weber[10] coined the expression MB factor (for Marsh-Bendall). Goodall and Szent-Györgyi,[12] I believe, were the first to use the term relaxing factor and, in the studies to be mentioned shortly, they also referred to an X factor; and Bozler[13] used the expression relaxation factor. This variety undoubtedly makes for some difficulty in writing about or discussing this problem, and I think a more general expression, such as relaxing factor system (RFS), might

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[*] Part of the work reported in the paper was carried out during the tenure of an Established Investigatorship of the American Heart Association, Inc. The details of the work on the isolation of the relaxing principle in a purified, soluble form were done in collaboration with F. N. Briggs and G. Kaldor, and were supported by Grants H-1166 (C5 and C6), H-2205, and H-3260 from the National Heart Institute, Public Health Service, Bethesda, Md., and by grants from The Muscular Dystrophy Associations of America, Inc., New York, N. Y., the Life Insurance Medical Research Fund, New York, N. Y., and the Massachusetts Heart Association, Boston, Mass. This work will be reported elsewhere; preliminary reports have been published.[1, 2]

be useful, although one runs the risk of introducing another new and perhaps unnecessary term.

This system, as is evident from the early work, has some interesting features, which immediately suggest various ways in which it could serve to control muscle activity. The relaxing system requires Mg^{++}, and is very strongly inhibited by Ca^{++}.[7,9] As shown by Hasselbach and Weber,[10] it works only at a certain supraoptimal ATP concentration and has no effect at lower ones. All of these effects appear in whatever assay system is used, that is, whether one studies the so-called syneresis of myofibrils, the contraction of muscle fiber bundles, or the ATPase activity of myofibrils. It should be noted for further discussion that very often conclusions about the RFS are based on experiments that do not directly show relaxation in the sense of inhibition or reversal of contraction; however, because of the close parallelism in these different systems, it might be concluded, for instance, from the inhibition of myofibrillar ATPase, that the relaxing factor was active in a particular experiment.

The work of Bozler[13] and that of Goodall and Szent-Györgyi[12] suggested that creatine phosphate and possibly some enzyme component may be involved in relaxation. This line of thought was followed up by Lorand[11] who, working with glycerinated fiber bundles, obtained relaxation on addition of creatine phosphate and the purified kinase to a bundle that had contracted under the influence of ATP. These observations, followed by Bendall's report,[14] according to which myokinase should be identical with the relaxing factor, and by the later report of Moos and Lorand[15] that phosphoenolpyruvate and the kinases presumably present cause relaxation, made it rather attractive to believe that the mechanism of relaxation was intimately related to the rephosphorylation of ADP, possibly bound to the structure of the contractile machinery.

All, however, was not plain sailing, for Kumagai *et al.*,[16] in Japan, found that in their system myokinase or the creatine phosphate-creatine kinase system did not produce relaxation unless a particulate component was also present, this particulate component having been identified tentatively with the Kielley-Meyerhof ATPase[17] granules; they found also that, depending on the age of the fibers, a third protein component was also necessary. The critical and careful work of Briggs and Portzehl[18, 19] made it clear that in the case of well-washed muscle fiber bundles the transphosphorylating systems did not produce relaxation unless another fraction, at that time merely called relaxing factor, was added. This relaxing factor may or may not be present in the muscle fiber bundles on which the experiments were carried out, the older and more throughly washed fibers having lost this factor. If this factor were absent, no amount of myokinase or creatine kinase could produce relaxation in either bundles or single fibers, and the apparent success of the transphosphorylating systems in causing relaxation of a fiber bundle was explained in terms of their maintaining a sufficiently high concentration of ATP[10] in the center of the fiber bundle, provided, of course, that the factor proper was present. Weber mentioned this in his presentation, and I only wish to reiterate the fact that in the case of single fibers, in contrast to fiber bundles, addition of purified kinases is not required for relaxation, since the ATP concentration throughout the fiber is apparently the same as that in the bath.

More recently Lorand and his colleagues[20] and Ebashi[21] published some observations on the effect of myokinase, creatine phosphokinase, and phosphoenolpyruvate on relaxation in the presence of the particulate component, also called granules, referred to in connection with the work of Kumagai *et al.*;[16] from this work it would appear that there is some potentiation of the granule effect by these enzyme systems. Portzehl,[22] however, studying inhibition of myofibrillar ATPase by essentially the same particulate muscle preparation, found no need for the addition of any transphosphorylating system. Her work was recently confirmed by Bendall,[23] who also found the granules to be effective in the original Marsh system; that is, in the inhibition of the syneresis of myofibrils in the presence of ATP.

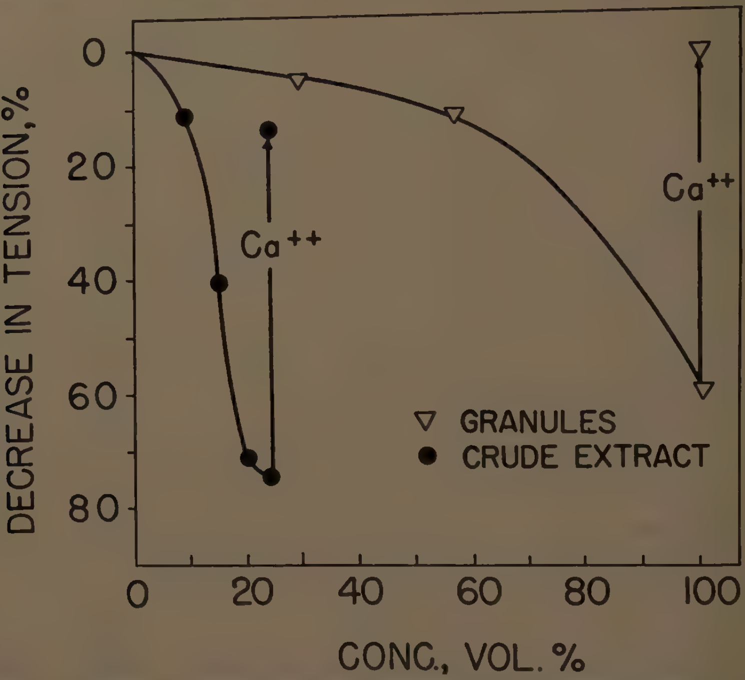

FIGURE 1. Influence of crude extract and granules on ATP-induced tension of single glycerinated muscle fibers. The concentration of extract is expressed in volume per cent. The concentration of granules (volume per cent) is expressed in terms of the volume of the crude extract from which they were obtained. Tension measurements were made on single fibers (60 μ thick) prepared from rabbit psoas muscle that had been extensively washed with 50 per cent glycerol and stored at −15° C. for 50 days. The bath contained 2 mM Mg^{++}, 5 mM ATP, 20 mM (hydroxymethyl)aminoethane (TRIS) buffer, *p*H 7.5; the ionic strength was adjusted with KCl to 0.16; total volume 4 ml. 23° C. The "crude extract" was prepared by blending rabbit muscle in 3 volumes of 320 mM sucrose, 20 mM histidine, 50 mM KCl and 2.5 mM oxalate, *p*H 7.5; centrifuged at 3600 g. The supernatant is referred to as crude extract. Further centrifugation followed at 35,000 g for 120 min., and the sediment is referred to as granules. The granules were taken up in 320 mM sucrose in 1/10 of the volume of the crude extract. Symbols: ●, crude extract; ▽, granules. Reproduced by permission from *Biochimica et Biophysica Acta.*[27]

It might be convenient to add at this point that, in conformity with the usage of others in this field, we have restricted the use of the term RFS to agents or systems isolated from muscle and causing relaxation in the presence of a roughly physiological concentration of ATP. These systems may play a physiological role, but reagents that had been known for some time to plasticize,[24] such as pyrophosphate and ATP in higher concentrations, or urea,[25, 26] would not come under this definition and probably their mechanism of action is quite different from that of the system discovered by Marsh.

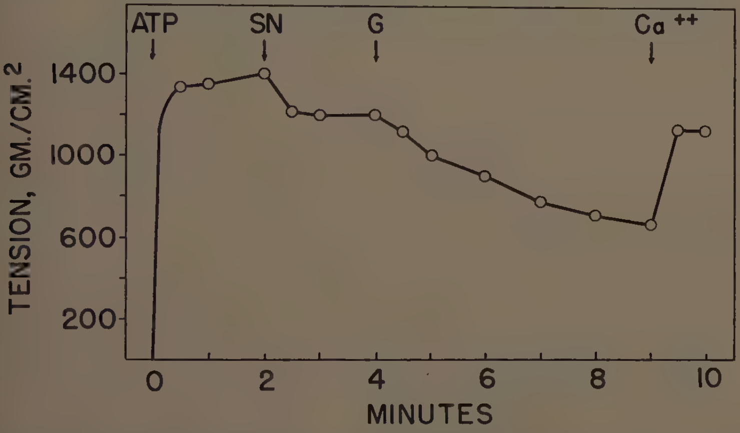

FIGURE 2. Influence of granules and supernatant, prepared from crude extract by centrifugation for 120 min. at 35,000 g, on tension. Granules were prepared as described in the caption to FIGURE 1. Fibers had been extracted for approximately 300 days. At 0 time the fiber was placed in the bath containing 5 mM ATP (for other components, see FIGURE 1). G indicates the addition of granules (final volume concentration, in terms of crude extract, 22 per cent); SN indicates the addition of supernatant (final volume concentration, in terms of crude extract, 22 per cent). At 10 min. Ca^{++} was added, final concentration 2 mM.

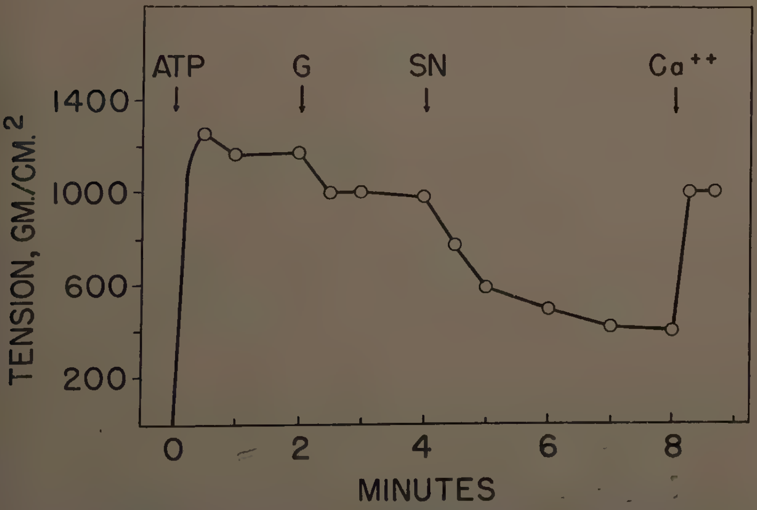

FIGURE 3. Additions as in FIGURE 2, except that the supernatant and granules were added in reverse order. For details see caption to FIGURE 1. Reproduced by permission from *Biochimica et Biophysica Acta*.[27]

This was, then, roughly the picture when Briggs, Kaldor, and I turned our attention to the isolation of the relaxing principle in a purified, soluble form,[1, 2] this being usually considered the crowning glory of a biochemist's endeavors. Our starting point was Portzehl's previously mentioned observation[22] that granules without addition of myokinase or creatine kinase inhibit myofibrillar ATPase and her conclusion that the granules alone accounted for the effect of a crude muscle extract. We decided to use, as our assay, tension measurements on single glycerinated muscle fibers and, to our great surprise, the granules, when used in a concentration that was effective in Portzehl's test, did not cause relaxation of these fibers. We found that one could achieve relaxation by increasing the amount of granules, but, if this effect is compared with that of the crude extract, it is clear that the granules are far less active than they would be if the activity of the crude extract were actually due to

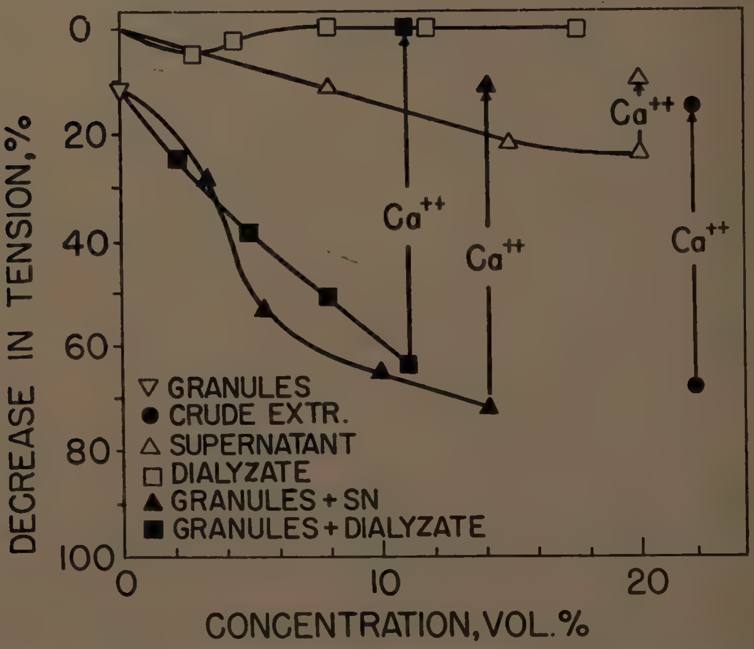

FIGURE 4. Influence of supernatant and its dialyzate with and without granules on ATP-induced tension of single fibers. The supernatant was dialyzed against an equal volume of the liquid used for extracting the muscle when preparing granules (see caption to FIGURE 1). In comparisons, correction was made for the dilution occurring in dialysis. Abscissa: concentration of supernatant or dialyzate in terms of the volume of the crude extract from which they were obtained. Without added granules: △, supernatant; □, dialyzate. With added granules: ▲, supernatant; ■, dialyzate. The granules were added in a concentration equivalent to 22 vol. per cent of crude extract. ● Indicates the effect of crude extract, 22 vol. per cent. Not shown in the figure is the fact that an amount of oxalate equivalent to the highest in these experiments did not have an activating effect on the granules in the absence of cofactor. By increasing the final concentration of oxalate to 2.5 mM (the concentration present in the ATPase experiments—see FIGURE 6) the amount of RFS (crude extract, granules, or granules plus cofactor) necessary for relaxation was reduced by a factor of 5 to 10. Arrows indicate reversal by 2 mM Ca^{++}. Fibers had been extracted for 71 days. For other details, see caption to FIGURE 1. Reproduced by permission from *Biochimica et Biophysica Acta.*[27]

them (FIGURE 1). If some supernatant is added to granules that were centrifuged out of the crude extract at 35,000 g, relaxation occurs (FIGURE 2). The amount of supernatant that produces this effect has a very small effect by itself (FIGURE 3).

If increasing amounts of the supernatant (SN) are added to granules obtained from an amount of crude extract that just causes maximal reduction of tension, the relaxing effect will be fully restored when the ratio of supernatant to granules corresponds to that in the parent extract (FIGURE 4). We soon discovered that the supernatant could be replaced by a dialyzate prepared from it, and the granules and dialyzate from the 35,000-g supernatant recombined in their original proportion were as active as the parent extract (FIGURE 5). This strongly suggests that, in addition to the granules, a dialyzable cofactor is involved in the relaxation mechanism. This cofactor, being dialyzable, is obviously not one of the protein fractions previously implicated, including transphosphorylating enzymes, and a further check showed that the substrates involved in these transphosphorylating systems, such as phosphoenolpyruvate or creatine phosphate, could not replace the cofactor. Relaxation in the reconstituted system is inhibited by Ca^{++}, as found originally for the Marsh factor. We also found that 2 mM carnosine is another inhibitor of the RFS, when assayed on both single fibers and myofibrils.

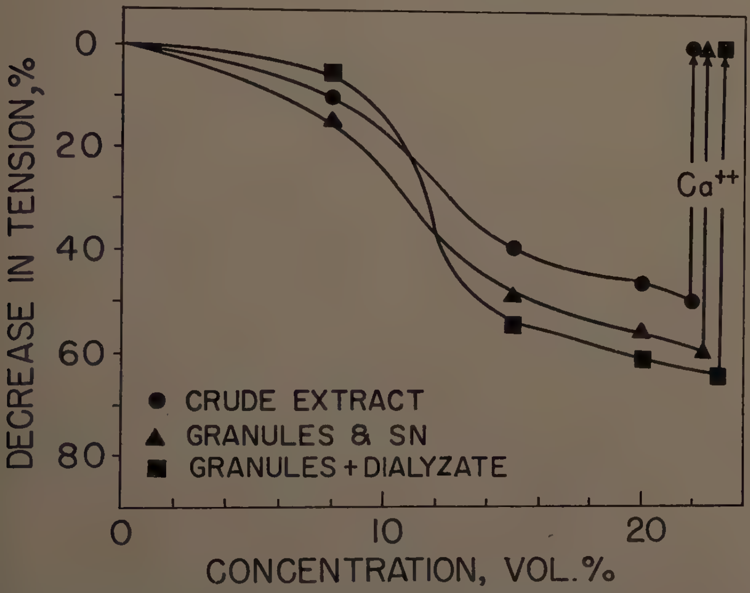

FIGURE 5. Relaxing activity of reconstituted systems on single fibers. ●, Crude extract; ▲, granules and supernatant mixed in the proportion in which they occur in the crude extract; ■, granules and dialyzate mixed in the proportion in which they occur in the crude extract. Concentrations as defined in the caption of FIGURES 1 and 4. Fibers had been extracted for 54 days. For other details, see the caption to FIGURE 1. Reproduced by permission from *Biochimica et Biophysica Acta.*[27]

The requirements for the cofactor could also be demonstrated in experiments with myofibrils, studying the inhibition of their ATPase by granules. Here we encountered essentially two types of preparations, and we still do not know why we sometimes get one and sometimes the other. In one case the pattern is essentially the same as with the single-fiber system; that is, granules are far less effective than the crude extract, and they can be brought to the original activity by adding the dialyzable cofactor (FIGURES 6 and 7). It should be noted that the activity of the granules cannot be increased beyond that of the original extract. With the other type of preparation, and this must have been the type on which the work in Weber's laboratory was done, the crude extract and the granules have almost the same activity (FIGURE 8).

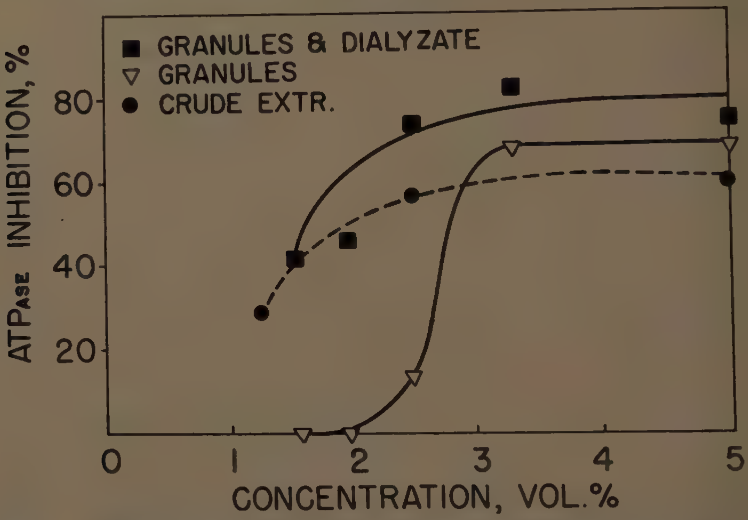

FIGURE 6. Inhibition of myofibrillar ATPase by granules requiring cofactor. The myofibrillar suspension was prepared by homogenizing in the Waring Blendor rabbit skeletal muscle with 5 volumes of 65 mM succinate buffer, pH 7.5, followed by centrifugation at 600 g. The sediment clearly showed two layers, the bottom one contained unbroken cells and connective tissue debris; the upper layer containing the myofibrils was resuspended in the buffer, and the centrifugation-resuspension procedure repeated 3 more times. The final fibril suspension contained about 2 mg. of N/ml. The "granules" and crude extract were prepared as described in the caption to FIGURE 1. The ATPase assay was carried in the medium used for the preparation of the granules in a total volume of 1 ml., containing 5 mM of ATP and 5 mM of $MgSO_4$. The basic system contained 0.1 ml. of myofibrillar suspension. The maximal phosphate splitting amounted to 1.5 μmoles in a 5-min. incubation at 23° C. Inorganic phosphate was determined, after deproteinization with trichloracetic acid, according to the Fiske-SubbaRow method. The concentration of crude extract is expressed in volume per cent. The concentration of granules or granules and dialyzate (volume per cent) is expressed in terms of the volume of crude extract from which they were obtained. Symbols: ●, crude extract; ▽, granules; ■, granules and dialyzate, mixed in the proportion in which they were present in the crude extract. Reproduced by permission from *Biochimica et Biophysica Acta*.[28]

If, however, one incubates granules of the latter type at 37° C. for 1 hour, they become far less active than the crude extract, but can now be reactivated by the addition of the cofactor (FIGURE 9). This experiment is open to several interpretations, although at present we have insufficient evidence on the basis of which we can distinguish between them. One possibility would be that the

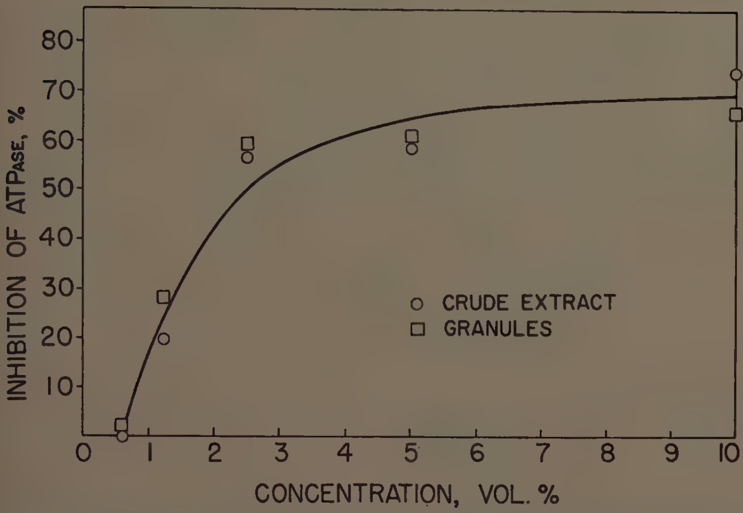

FIGURE 7. Inhibition of myofibrillar ATPase by granules not requiring cofactor. For details, see caption to FIGURE 6. Symbols: □, granules; ○, crude extract. Reproduced by permission from *Biochimica et Biophysica Acta*.[28]

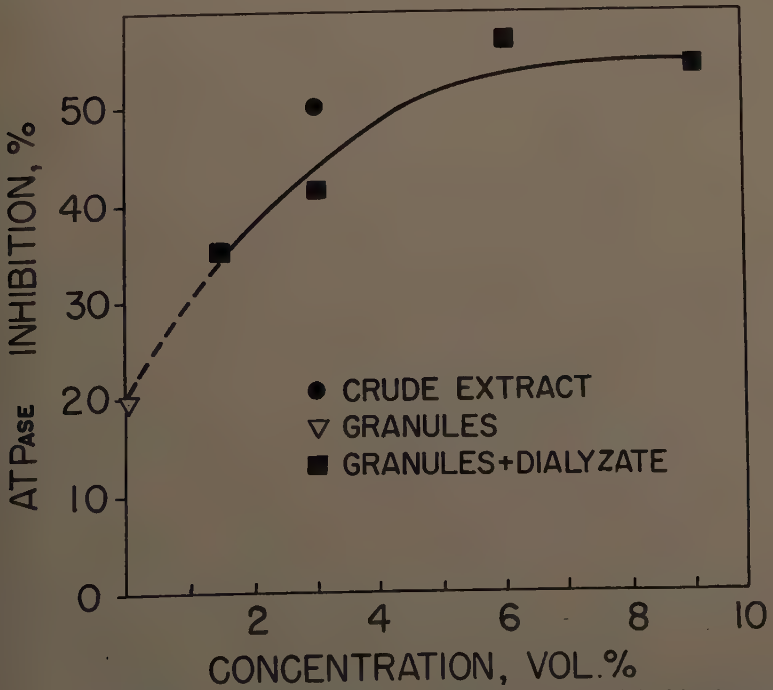

FIGURE 8. The influence of increasing amounts of dialyzate on the inhibition by granules of myofibrillar ATPase. For the meaning of concentration see FIGURE 6. Symbols: ●, crude extract; ▽, granules; ■, granules and dialyzate. For other details see the caption to FIGURE 6. Reproduced by permission from *Biochimica et Biophysica Acta*.[28]

cofactor is normally bound to the granules, but that heating causes a dissociation of the cofactor by altering the properties of the granules, and that the original complex can be restored only by adding cofactor in excess. Another way of regarding this phenomenon is to postulate that normal granules do not require a cofactor, and that a cofactor normally present in the muscle but dissociated from the granules, when added to such heated granules, restores their structural and/or functional integrity. On either of these views, the fact that there are granule preparations that require a cofactor even when unheated in the case of myofibrillar ATPase experiments (granules always require a cofactor in the case of single-fiber experiments provided their concentration is sufficiently low) could be explained by assuming that the same sort of change that occurs in an exaggerated form during heating occurs also, to a smaller or larger extent, in the course of the preparative procedure.

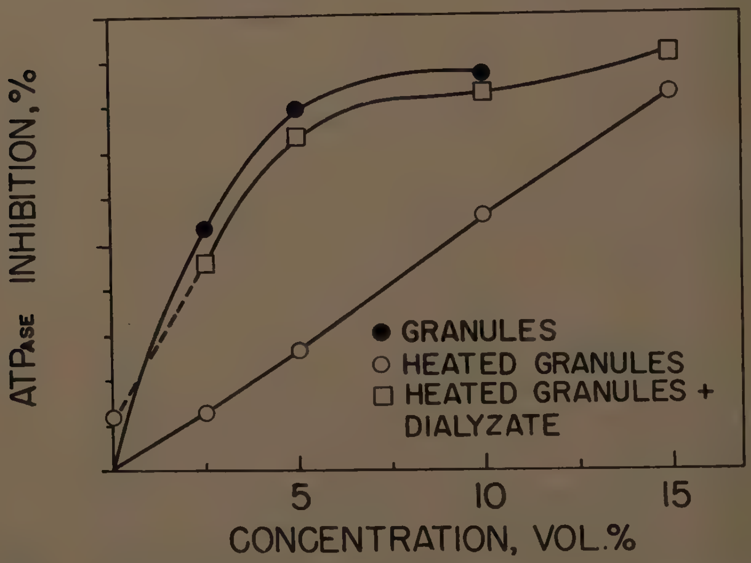

FIGURE 9. The inhibition of myofibrillar ATPase by heated granules. For the meaning o concentration, see FIGURE 6. Symbols: ●, granules; ○, granules incubated at 37° C. for 60 min.; □, heated granules and dialyzate mixed in the proportion in which they were presented in the crude extract. For other details, see caption to FIGURE 6. Reproduced by permission from *Biochimica et Biophysica Acta.*[28]

I now mention some facts relating to the properties of the granules and of the cofactor. As my earlier remarks have indicated, we were unable to solubilize the granular component; indeed, from some experiments with sonic vibration it appears that a certain structural integrity of the granules is required, since sonic vibrations destroy the activity of the granules even in the presence of cofactor. Agents known to interfere with lipid structure, such as desoxycholate, also destroy the activity of the granules, and thymol, another lipid-soluble substance, was likewise found to destroy the activity.

Turning now to the cofactor, let me repeat that substrates of transphosphorylating enzymes cannot replace it. We found, however, that pyrophosphate in a concentration of 2 mM, which by itself is ineffective on the fibers or fibrils, reactivates cofactor-requiring granules (FIGURES 10 and 11). This observation recalls the findings of Bendall,[30] according to which fairly fresh glycerinated muscle fibers can be relaxed with pyrophosphate and ATP. In the light of our observations, Bendall's results are most naturally interpreted by saying that his fibers still contained the granular component, but lacked the cofactor, and that addition of pyrophosphate activated the granules. The cofactor cannot, however, be identical with pyrophosphate, since pyrophosphatase inhibits the pyrophosphate effect, but not that of the cofactor (FIGURES 12 and 13); other properties of the cofactor also seem to rule out this identity.

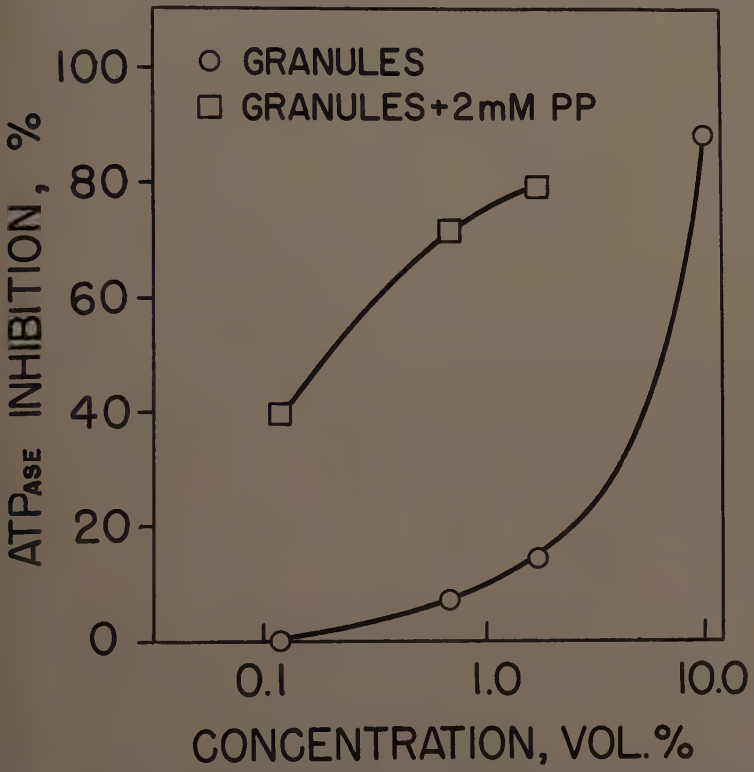

FIGURE 10. The effect of pyrophosphate on the inhibition by granules of myofibrillar ATPase. Abscissa: concentration of granules in terms of an equivalent volume of crude extract. Symbols: ○, granules; □, granules 2 mM pyrophosphate. For details of the assay system, see caption to FIGURE 6. Reproduced by permission from *Biochimica et Biophysica Acta*.[29]

The cofactor is rather labile; TABLE 1 shows what it can and cannot withstand. It appears to be destroyed fairly easily at both acid and alkaline *p*Hs, and is moderately heat-labile. A useful property for further purification may be its absorption to charcoal and to Dowex-1.

Let us now consider briefly the problem of the mechanism of the RFS. In the absence of detailed information on the active components of the granules

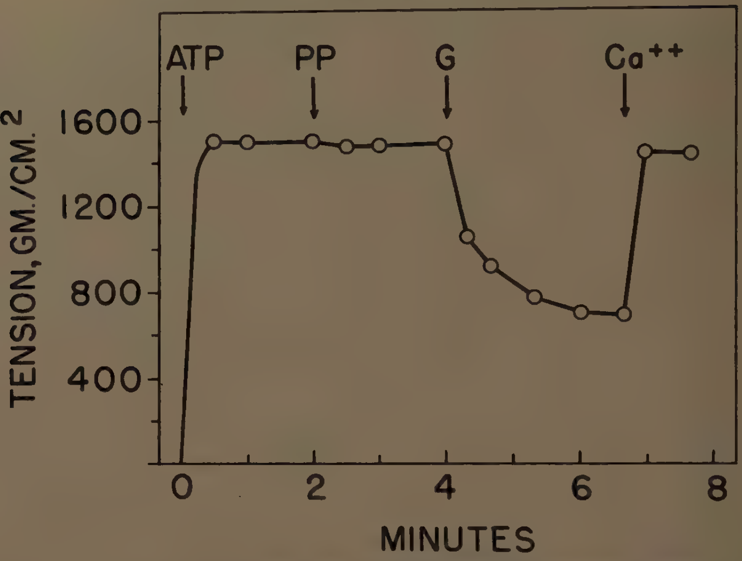

FIGURE 11. Activation of the relaxing activity of granules by pyrophosphate and 36,000-g supernatant. Fibers had been extracted with 50 per cent glycerol for approximately 300 days. At 0 time the fiber was placed in a bath containing 5 mM ATP, 2 mM $MgCl_2$, 20 mM TRIS and 70 mM KCl. At the first arrow addition of pyrophosphate, final concentration 2 mM; G indicates the addition of granules (final volume concentration in terms of crude extract, 22 per cent). At 7 min. Ca^{++} was added, final concentration 2 mM. Reproduced by permission from *Biochimica et Biophysica Acta.*[29]

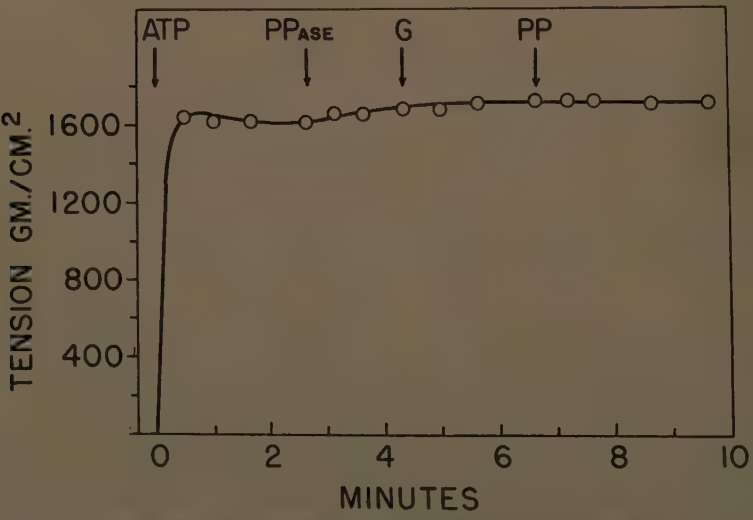

FIGURE 12. Conditions as in FIGURE 11, except that pyrophosphatase was added before the granules and pyrophosphate. Reproduced by permission from *Biochimica et Biophysica Acta.*[29]

and on the nature of the cofactor, it is rather difficult to theorize about this. It appears to be quite reasonable to assume, in line with suggestions made by several workers in this field (Bendall,[14] Lorand *et al.*,[20] and Weber[5]) that in the last analysis the relaxation is brought about by a substance elaborated by the RFS in the presence of ATP. Since no relaxing substance can be detected in a granule-free system, one must assume that either the hypothetical substance

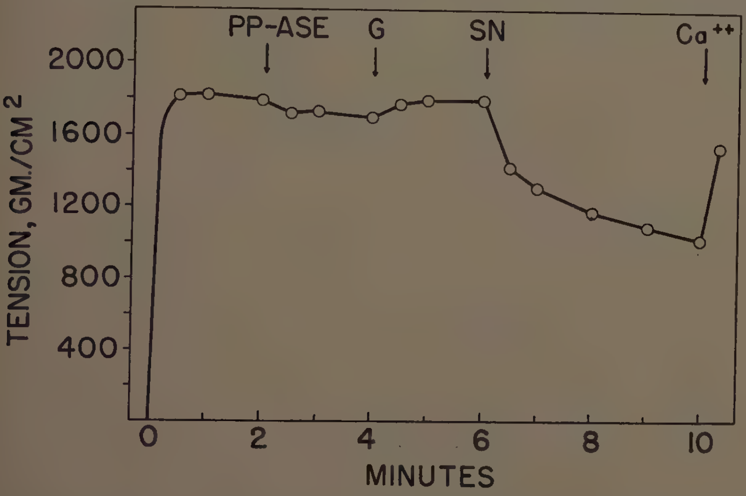

FIGURE 13. Conditions as in FIGURE 11, except that 35,000-g supernatant (SN) was added instead of pyrophosphate. Reproduced by permission from *Biochimica et Biophysica Acta.*[29]

TABLE 1
THE EFFECT OF VARIOUS TREATMENTS ON DIALYZATE ACTIVITY

Experiment	Inhibitions of myofibrillar ATPase, percentages
Granules incubated at 37° C. for 60 min.	5
1. Plus nontreated dialyzate	69
2. Plus acid-treated dialyzate (pH 3)	10
3. Plus alkali-treated dialyzate (pH 10)	20
4. Plus Dowex-1-treated dialyzate	22
5. Plus charcoal-treated dialyzate	0
6. Plus dialyzate heated at 60° C. for 10 min.	44
7. Plus dialyzate boiled for 10 min.	21
Dialyzate	0

The amount of dialyzate used was just sufficient to produce maximal inhibition of the myofibrillar ATPase when added to granules that had been treated at 37° C. for 60 min. The acid- and alkali-treated dialyzates were kept at the pH indicated for 30 min. at 0° C. and were neutralized with a small volume of 0.25 N alkali or acid before testing. Dowex-1 was in the chlorine form; no change of pH of the dialyzate was observed as a result of the treatment with it. The charcoal was thoroughly washed with HCl and water. For other details of the assay see the caption to FIGURE 7. Reproduced by permission from *Biochimica et Biophysica Acta.*[28]

is passed on directly to the myofibrils or that the myofibrils participate in its production.*

In the course of our efforts to screen various normal cell constituents for cofactor activity, however, we have encountered a substance, namely pyridoxalphosphate, whose effect resembles that of the complete RFS.[32] Pyridoxalphosphate inhibits myofibrillar ATPase (FIGURE 14); it also inhibits the syneresis of myofibrils (TABLE 2) and, what is perhaps most interesting, the inhibition of ATPase appears to depend on the ATP concentration in a way reminiscent of the ATP dependence of the RFS itself: the inhibition is greatest at the ATP concentration at which the uninhibited myofibrillar ATPase is most active. This, taken together with the fact that carnosine inhibits this effect (FIGURE 14), just as it inhibits the effect of the RFS, may indicate that pyridoxalphosphate, if itself not directly involved in the relaxing mechanism, may be a model substance for whatever hypothetical relaxing compound may be produced by the RFS. The fact that the physiological role of carnosine may be related to the inhibition of the relaxing factor system is supported by some observations of Hayashi[33] in Japan that intracellular injection of carnosine causes contraction. This might be due to the inhibition of the RFS, thereby leading to the activation of the contractile machinery. It should be pointed out that all our efforts to show that carnosine functions as a phosphate acceptor from ATP or pyridoxalphosphate failed to do so.

TABLE 2

THE EFFECT OF PYRIDOXALPHOSPHATE AND CARNOSINE ON THE SYNERESIS OF MYOFIBRILS

Carnosine, mM	Pyridoxalphosphate, mM					
	0.5	1	2	3	4	5
0	++++	++++	−	−	−	−
2	++++	++++	−	−	−	−
4	++++	++++	+	+	−	−
6	++++	++++	++++	++	−	−
8	++++	++++	++++	+++	+	−
10	++++	++++	++++	++++	+	+
12	++++	++++	++++	++++	++	+
14	++++	++++	++++	++++	+++	++
16	++++	++++	++++	++++	++++	++
18	++++	++++	++++	++++	++++	+++
20	++++	++++	++++	++++	++++	++++

The myofibrillar suspension contained 10 mM Mg^{++}, 50 mM succinate, *p*H 7.5, 5 mM ATP, and 2.1 mg. protein/ml. Pyridoxalphosphate and carnosine concentration as given in the table. The degree of syneresis was judged on an arbitrary scale, ++++, indicating complete settling out of the myofibrils. The experiments were carried out at room temperature, 23 to 23° C., and were read 3 min. after addition of ATP. From Kaldor and Gergely.[32] Reproduced by permission from the *Archives of Biochemistry and Biophysics.*

Consideration of the experimental facts suggests that a soluble relaxing substance, if formed at all, is destroyed at about the same rate at which it is produced; otherwise it should slowly accumulate in fibers or myofibrils, and one would expect the same degree of relaxation regardless of the amount of RFS, provided one waited long enough. That this is not the case is particularly clear from experiments with single fibers. In his contribution to this publication Weber suggests that the cofactor's role is to prevent the destruction by the myofibrils of the "relaxing substance." On this view it is difficult to see why, in their ATPase inhibition, some granules are at all concentrations as active as the parent crude extract and cannot be stimulated by the addition of the cofactor, for, if the role of the cofactor were to inhibit the destruction of a relaxing substance produced by the granules, addition of the cofactor should increase the apparent activity of the granules at suboptimal concentrations. The finding that granules can never be stimulated beyond the activity of the crude extract also speaks against such a role of the cofactor. It appears more reasonable to assume either that the cofactor acts on the granules maintaining their structural integrity or that it is required by the granules for the production of the relaxing substance.

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* Since the completion of this manuscript Parker and Gergely were able to show that the incubation of granules, ATP, and cofactor leads to the formation of a proteinfree substance that, after the removal of the granules, reproduces the effect of the complete RFS.[31]

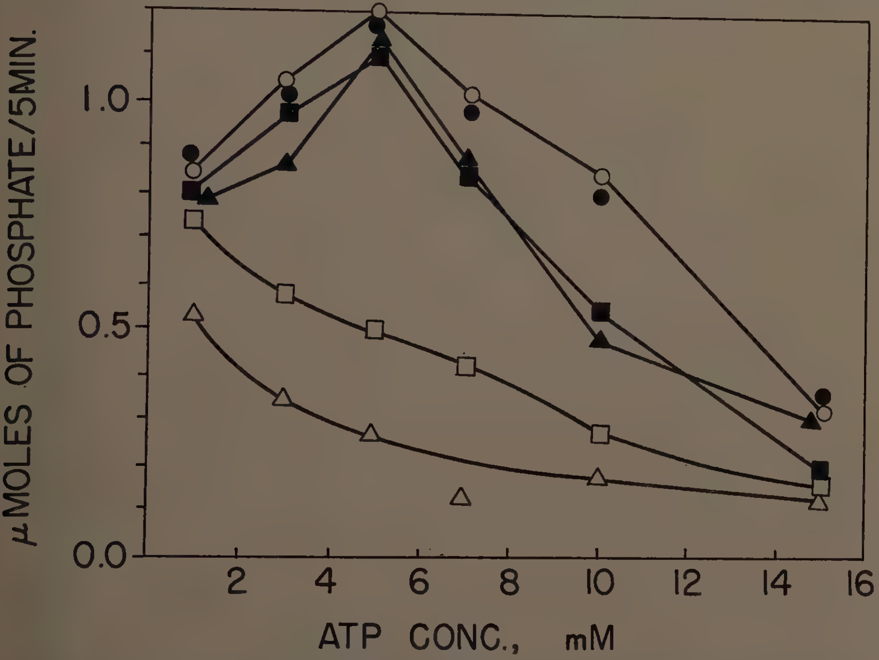

Figure 14. The dependence of myofibrillar ATPase activity on ATP concentration at various pyridoxalphosphate and carnosine levels. The incubation mixture contained 10 mM Mg^{++}, 50 mM succinate, and 0.65 mg. of myofibrillar protein in a total volume of 1 ml., *p*H 7.5. ATP concentration on abscissa. Other additions as indicated below: ○, without pyridoxalphosphate and carnosine; □, 1 mM pyridoxalphosphate; ■, 1 mM pyridoxalphosphate and 5 mM carnosine; △, 2 mM pyridoxalphosphate; ▲, 2 mM pyridoxalphosphate and 10 mM carnosine; ●, 10 mM carnosine. For other details, see caption to figure 6. Reproduced by permission from *Archives of Biochemistry and Biophysics*.[32]

Finally, there arises the question of how the relaxing factor system fits into the contraction-relaxation cycle of muscle. Weber's suggestion that the relaxing factor system causes dissociation of the actomyosin system, thereby producing myosin having a lower ATPase activity, is very attractive. At present there is no direct experimental evidence that such primary dissociation occurs, and it would be highly desirable to obtain it. In the meantime I think one must entertain the possibility that, in the conditions of an organized fibril, the primary function of the relaxing factor system is to inhibit the ATPase of actomyosin, thereby secondarily increasing the average occupancy of the enzymatic sites by ATP and, if the *in vitro* findings[34] also hold *in vivo*, causing a dissociation of the actomyosin filaments to produce relaxation. Such a theory has been worked out in detail by A. F. Huxley.[35]

The fact that the effect of the RFS can be inhibited by either Ca^{++} or carnosine or by withdrawal of Mg^{++} offers several levers by which the onset of stimulation could put the relaxing factor system out of action, so that, indeed, the initiation of contraction would be analogous, as I suggested at the outset, to taking one's foot off a decelerator. We still do not know whether it is contraction or relaxation that is under primary control, but it is clear that further studies on the RFS may throw more light on this fascinating problem.

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EARLY KINETICS OF THE CYTOCHROME *B* RESPONSE TO MUSCULAR CONTRACTION

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Elsewhere in this monograph Chance discusses some aspects of the cytochrome system in intact muscle; he correlates the contractile and the oxidative activities of the tissue. Following the fundamental work of Chance and Connelly (1957) and of Weber (1957), an attempt was made to determine the time relations between the mechanical events of muscular contraction and the response of the cytochrome chain. I shall briefly report on the results.

Information on the moment of the involvement of the hydrolysis of adenosine triphosphate (ATP) to adenosine diphosphate (ADP) could have an important bearing on various theories of muscular contraction. Specifically, some information on the possible role of ATP as the immediate energy donor for the contraction could be expected, while the question of the direct involvement of an ATPase during the contraction or the relaxation phase might be elucidated.

The interpretation of the expected data depends on two assumptions. The first of these is the question of the specificity of ADP as the initiator of oxidation-reduction changes of the respiratory chain. As this assumption is discussed extensively by Chance in this publication, it may suffice to mention here that, of all the physiologically occurring substances tested thus far, only ADP and inorganic phosphate (P_i) will produce these specific changes in the oxidation-reduction levels of the cytochromes. In this respect the effectiveness of ADP is twenty times that of P_i.

The second assumption concerns the rate of diffusion of molecules in the intracellular milieu. This point is essential in the calculation of the results and may, therefore, better be deferred to that section. In addition, it should be mentioned that the speed of the reaction of isolated mitochondria with added ADP is measurable in a few milliseconds (Chance, personal communication, 1957).

For measurement of the small light-absorption changes involved, the double-beam spectrophotometer of Chance was employed. This type of spectrophotometer is specifically designed to detect wave-length-specific absorption changes in the presence of larger wave-length-nonspecific changes. In the present case, this type of instrumentation is necessary to cancel the various light diffraction, absorption, and scattering phenomena that are known to occur during the contraction (Sandow, 1936; Buchthal and Knappeis, 1940; Hill, 1949, 1953).

To reduce the gross movements in the optical path, the muscle is stretched to approximately 110 per cent of its rest length over a slightly curved Lucite sheet. One end of the muscle is securely attached to the frame, while the other is fastened to a thin Lucite beam bearing strain gauges for the recording of isometric tension. To reduce the nonspecific light transmission effects further, the window of the end-on photomultiplier is brought to within a few millimeters of the muscle. This increases the solid angle subtended by the photomultiplier and thereby increases the amount of scattered and diffracted light reaching the photosensitive surface.

The preparation is immersed in rapidly flowing Ringer's solution oxygenated with 100 per cent O_2. Stimulation is by square waves or by condenser discharges at 2 cathodes adjacent to the optical window. In the toad's sartorius muscle this arrangement provides simultaneous stimulation close to the pelvic end and at approximately the point of entrance of the nerve. The optical window has the form of a rectangle with a width of approximately two thirds that of the preparation. The length of the optical path through the muscle is equal to its thickness; that is, about 1 mm.

The tension, stimulus marker, and spectrophotometric traces are recorded simultaneously on a film-recording oscillograph equipped with rapidly responding galvanometers.

The experiments reported here were performed on the sartorius muscle of the toad (*Bufo marinus*). This preparation is especially suited to our purposes, because of the parallel arrangement of the fibers, the high cytochrome content, and the fact that this muscle does not contain myoglobin (hemoglobin is removed by perfusion of the animal with Ringer's solution). The relative slowness of the contraction also aids in the separation of the various events during the twitch.

The typical response to a twitch is an oxidation of cytochrome *b*. This oxidation reaches a maximum in a few seconds, to become reduced again in a matter of a few minutes. FIGURE 1 shows the early part of the response. The oxidation-reduction level of cytochrome *b* is measured as the difference in absorption between 430 mμ (the wave length of the absorption peak of cytochrome *b*) and 410 mμ (reference wave length).

This type of record shows a clear difference between the time relations of the spectroscopic and the mechanical events. Until the peak tension has been reached, the spectroscopic trace exhibits only slight oscillations that are thought to be artifacts caused by the wave length nonspecific effects. The time lag between the stimulus and the oxidation of cytochrome *b* in this case is 220 msec.

The negligible deflections in the spectroscopic trace during the contraction phase show the extent to which the interference from the mechanical events has been reduced. Confirmation of our contention that cytochrome *b* is oxidized as a result of the twitch comes from 3 simple control experiments. In the first, the 2 wave lengths are set at 430 mμ. The effect of 1 twitch is then limited to a few small oscillations in the spectroscopic trace. The same result is produced at 430 minus 410 mμ. This shows the successful elimination of wave-length nonspecific optical effects. In the second experiment the reference wave length is kept constant, while the measuring wave length is varied from 410 to 450 mμ for a series of twitches. This produces a set of records from which the spectrum of this event can be plotted. The resulting curve (the "action spectrum") clearly shows a sharp maximum at 430 mμ corresponding to cytochrome *b*. In the third control experiment, recording is again at 430 minus 410 mμ, but the cytochrome response is eliminated by complete anoxia. Under such conditions the trace again shows small oscillations during the entire twitch, but no contribution from cytochrome *b*. We may therefore conclude that the deflection starting rather suddenly at the end of the contraction phase is truly the response of the cytochrome chain.

Identical measurements have been made also on the frog sartorius and the turtle coracohyoideus muscles (Jöbsis and Chance, 1957). In all these preparations, it was found that the beginning of the response of the cytochrome chain did not correlate with the beginning of the contraction phase, but was more closely associated with the peak of the twitch tension. Among the various preparations, the onset of the cytochrome *b* oxidation was found to vary from the end of the contraction phase (developed tension >80 per cent) to the beginning of the relaxation phase (remaining tension >80 per cent). The range of the delay between the onset of the contraction and the onset of the spectroscopic response is from 60 to 250 msec. in the various preparations and at various temperatures.

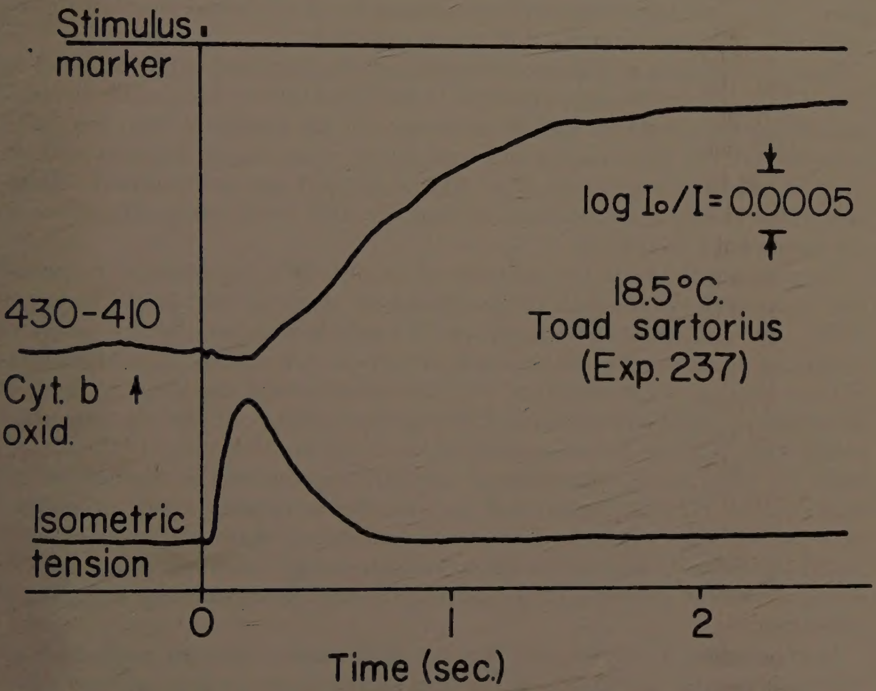

FIGURE 1. The response of cytochrome *b* to a single twitch.

Before considering the implications of these findings, the possible contribution of the time required for the diffusion of ADP from its site of formation to the mitochondria should be considered. From measurements of many photomicrographs of frog and toad skeletal muscle the distance between the edge of the myofibril and the mitochondrion was found to be smaller than 0.1 μ and probably of the order of 0.01 μ. No accurate figure for the diffusion coefficient of ADP in the cytoplasm has come to our attention, but the use of the figure for ATP under these circumstances is justified. This practice should introduce an error toward longer diffusion times and should therefore increase the margin of safety. A value of 2.5×10^{-8} sq. cm./sec. as determined by Hasselbach (1952) for slices of glycerinated muscle will be used.

From Fick's equation for the case of one-dimensional diffusion it can be calculated that the time required for the diffusion of ADP over a 0.1 μ distance should give us a half time of 5 msec. In other words, if the ADP concentration in the myofibrils is raised stepwise a given amount, the concentration at the mitochondria has reached one half that amount in 5 msec. Making an assumption of a distance of 0.01 μ and a value of 25 per cent equilibration, a level that should already be easily detectable, the time interval becomes less than 0.1 msec. It is clear that, even with the assumption of this nonfavorable diffusion coefficient, the time taken for diffusion is negligible.

Some implications of these results remain to be discussed. The observation that ADP does not become available to the cytochrome chain until the peak tension has been reached enables us to rule out the possibility that free ADP is formed at the beginning of the contraction phase or concomitant with it. This does not necessarily mean that ATP is not split during this period. However, it does imply that, if such is the case, the ADP is not freely diffusible until the onset of the relaxation.

Another possibility is the splitting of an unknown high-energy compound that is restored via the high-energy phosphate stores in the muscle, including ATP. For such a system to be efficient, it would be necessary for this unknown compound to be in rapid equilibrium with those $\sim$P stores. The abruptness of the initiation of the oxidation of cytochrome *b* would also speak in favor of an extremely rapid equilibration, if this scheme occurs. If the unknown compound were available for rephosphorylation at the very beginning of the twitch, while the delay in the appearance of the ADP was due to the slowness of the equilibration, then the initiation of the cytochrome response would necessarily be a gradual one. Therefore, the same conclusions outlined above for ADP would be relevant, that is, the unknown high-energy compound is not split or does not become available for reconstitution until approximately the onset of relaxation.

In conclusion, it may be said that our data favor a reaction mechanism involving either the adsorption of the high-energy donor to the contractile protein during the contraction phase or the involvement of such a donor in the relaxation process.

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